Studies on New Dehydropeptidase Inhibitors from Streptomyces(Dissertation_全文)

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Studies on New Dehydropeptidase Inhibitors from *Streptomyces*

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Contents

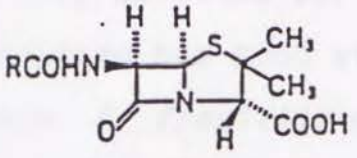
Chapter I Introduction

Over the past 20 years, many antibiotics have been synthesized by using the penam and cephem structures. When it was discovered, thienamycin was the first example of a novel, naturally occurring β-lactam with a carbapenem ring structure. Since then, other bicyclic and, more recently, monocyclic semisynthetic and synthetic compounds have been described. The carbapenem group of compounds differs from the penicillins (penams) in that the 1-sulfur atom is replaced by carbon and there is a double bond at positions 2 to 3 (Fig. 1).

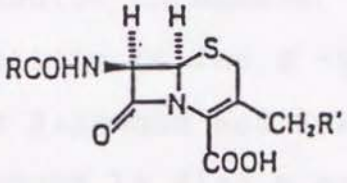
Carbapenems were discoverd almost simultaneously by workers at Beecham Research Laboratories and Merck Sharp & Dohme Research Laboratories. The former identified three compounds, MM4550, MM13902 and MM17880 from a culture of *Streptomyces olivaceus*[1]. These compounds were designated olivanic acids. Merck Sharp & Dohme Research Laboratories isolated thienamycin from a culture of *Streptomyces cattleya*[2]. Since then, 40 or more compounds have been described.

There is considerable information on a wide range of thienamycin derivatives. Thienamycin has a very broad antibacterial spectrum, including *P. aeruginosa*. Members of the family *Enterobacteriaceae* including many isolates resistant to other β-lactams, *S. aureus* and *Bacteroides fragilis* are also suspectible to thienamycin. However an early observa-

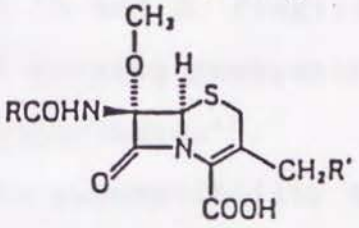
Fig. 1. β -Lactam antibiotics produced by microorganisms.



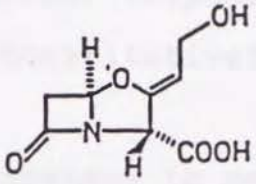
Penicillin (1928)



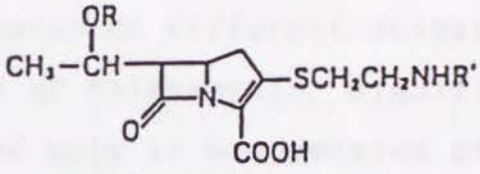
Cephalosporin (1953)



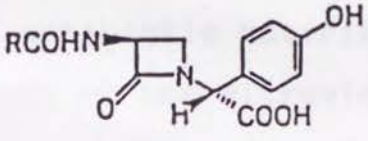
Cephamycin (1969)



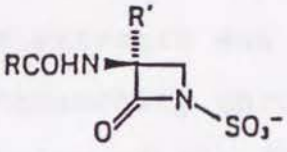
Clavulanic acid (1976)



Thienamycin (1976)



Norcardicin (1976)



Monolactam (1981)

tion[2] indicated the chemical instability of this compound.

Due to the chemical instability of thienamycin, futher development of it has ceased and a search for chemically stable derivatives was undertaken, investigating several hundred analogs. *N*-Formimidoyl thienamycin (imipenem, Fig. 2) was finally selected for studies in humans.

Imipenem has good stability to the β-lactamases from *S. aureus*, *B. fragillis* and Richmond and Sykes type Ia, Ⅲ and IVc enzymes[3]. The compound is also a potent inhibitor of type Ia and *B. fragillis* enzymes. Imipenem is active against strains possessing the constitutively produced P99 cephalosporinases[4].

The susceptibility of carbapenems to metabolic inactivation in animals has been a major problem. Kropp *et al.*[5] surveyed homogenates of different animal organs for enzymatic inactivation of thienamycin. Significant rates of degradation were found only in homogenates of the kidney, a finding consistent with the conclusions of disposition studies. Loss of antibiotic activity in extracts was accompanied by extinction of the ultraviolet-absorbing chromophore of the antibiotic, a hallmark of scission of the β-lactam bond (Fig. 3). The observation that the degrading enzyme was membrane-associated and zinc-dependent led to a review of the distribution and properties of known renal metallopeptidase. Most similar was the renal dipeptidase which had originally been designated dehydropeptidase-I by its discoverer Greenstein[6]. The product of enzyme-catalysed inactivation was

Fig. 2. Structures of imipenem and cilastatin.

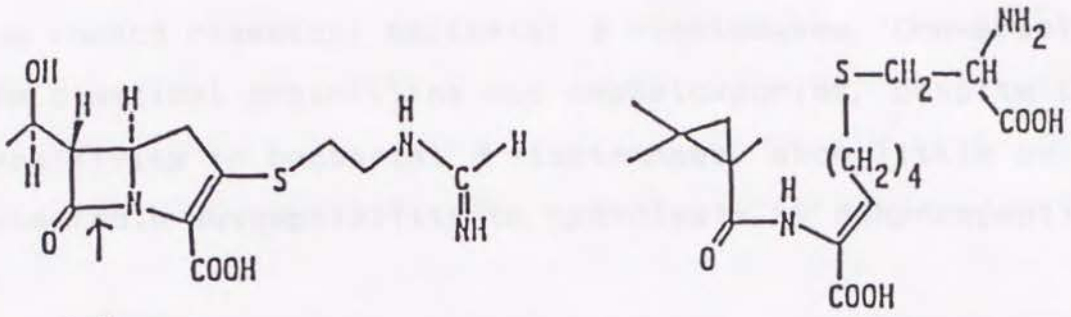
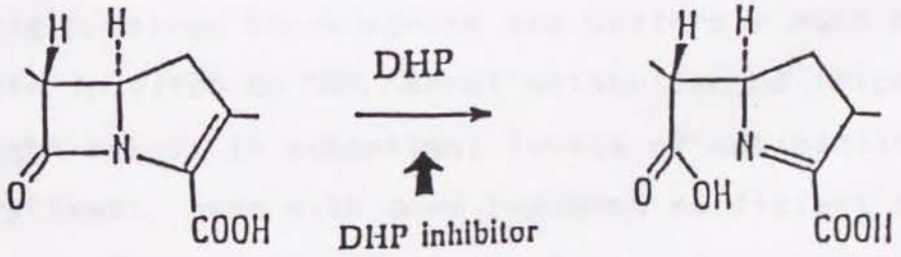


Fig. 3. Inactivation of carbapenem antibiotics by dehydropeptidase-I (DHP).



proved identical by NMR and mass spectroscopy with that generated by controlled acid hydrolysis of the β-lactam. Dehydropeptidase-I thus acts as anunprecedented mammalian β-lactamase upon thienamycin antibiotics, all the more remarkable considering the stability of thienamycin and imipenem toward classical bacterial β-lactamases. Conversely, the classical penicillins and cephalosporins, despite their sensitivity to bacterial β-lactamases, show little or no detectable susceptibility to hydrolysis by dehydropeptidase-I.

The enzyme responsible for this inactivation has been thus shown to be a renal tubular brush border dipeptidase (EC 3.4.13.11), dehydropeptidase-I (DHP).

Following administration of radiolabelled imipenem to the rat, rabbit and more recently in man, at least 90% of the administered radioactivity was found in the urine either as intact antibiotic or a fraction identical in its chromatographic mobility to the product of DHP catalysed hydrolysis DHP's role in metabolism readily explained the very low urinary recovery of *N*-acylated carbapenems of natural origin, since these agents are uniformly much more susceptible *in vitro* to DHP. Renal metabolism of imipenem in man might result in suboptimal levels of antibiotic in the urinary tract, even with dose regimens sufficient for the treatment of systemic infection. For example, in an individual with a 10% urinary recovery, concentrations of imipenem in the urine would fall below any desired level approxima-

tely 3 h earlier than those achieved by another non-metabolized antibiotic.

There have been two approaches to solve this problem. First, there is the structural approach. Nonbasic acylaminoalkyl-thio side chains at C-2 are less stable than other substituents. Appropriate alterations at this site provide relatively more stable compounds. Alteration at C-3 in the PS series have been shown to affect the stability to the dehydropeptidase of rat kidneys. The second approach is to combine the DHP inhibitor with the antibiotic. The latter approach developed an inhibitor of DHP suitable for co-administration with imipenem.

In a directed search for potential inhibitors, compounds resembling or containing the dehydropeptide bond were screened *in vitro* against DHP. Benzoyl-2-aminocrotonate was found to have moderate inhibitory activity. Systematic modification of this lead by Ashton *et al.*[7] resulted in the discovery of a class of 2,2-(+)-dimethylcyclopropylcarboxy-2-amino-3-alkyl-(Z)-propenoates with the greater inhibitory activity against both porcine and human renal DHP. These inhibitors are competitive and reversible in their action and showed a high degree of specificity. For example, at concentration up to 1 mM (10,000 times their *Ki* versus DHP), little or no inhibition was detected with the following zinc-metallopeptidase: acylase-I (hog kidney), carboxypeptidase-A (bovine pancreas), carboxypeptidase-B (bovine pancreas) and angiotensin-converting enzyme (rat lung). These com-

pounds are also devoid of antimicrobial activity when tested at the highest concentrations likely to be achieved in either blood or urine. Renal metabolism was inhibited for the longest duration by two analogues, the octenoate (MK0789) and the L-cysteinyl-thio-hexenoate (cilastatin, Fig. 2).

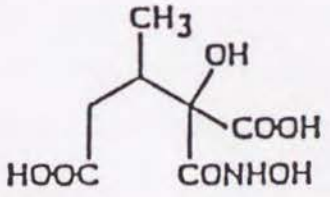
The importance of having a metabolically stable compound is twofold. First, the urinary recovery and the levels in urine will be greater. Second, the nephrotoxic breakdown products of imipenem adminstered will be reduced.

The effect of co-administered cilastatin on the urinary recovery, plasma profile and renal clearence of imipenem was determined in animals[8] and human[9]. The anticipated efficacy of cilastatin was observed in all tests. The augmented protective effect of imipenem co-administered with cilastatin was also verified in the animal models of bacterial infection. Moreover, cilastatin co-administered with imipenem prevents the occurrence of actue proximal tubular necrosis which is induced in rabbit kidney by an only imipenem administration.

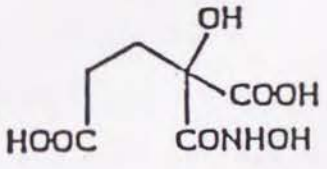
A rationale for the combination of imipenem with cilastatin can be compared with that for the two already established combination antimicrobials, trimethoprim/sulphamethoxazole and amoxicillin/clavulanate. In the latter two cases, both components are directed against bacterial metabolic processes. Incremental benefit is achieved by mutual potentiation of antibacterial activity against that fraction of organisms insensitive to either component or by restora-

tion of activity against a fraction strains exhibiting pre-existent resistance to one of the components. In the present case, however, imipenem is the sole antimicrobial principle. Its breadth of spectrum, potency and lack of cross-resistance with other antibiotics leaves little that could be improved upon by a co-administered antimicrobial partner. Cilastatin is instead directed to the control of antibiotic inactivation. This role of cilastatin is closely analogous to that of carbidopa in the carbidopa/levodopa combination. In both cases, the active ingredient is spared from universally present and undesirable metabolism and can achieve efficacy at a lower dose with attendant economic and safety benefits.

The validity of DHP inhibitors in the combination with carbapenem prompted the auther to search new ones for developing carbapenem antibiotics. Many enzyme inhibitors have been found from the microbial origin. The author was also able to find new DHP inhibitors shown below from the fermentation broth. It is the first ones obtained as a natural product.



WS1358A1



WS1358B1

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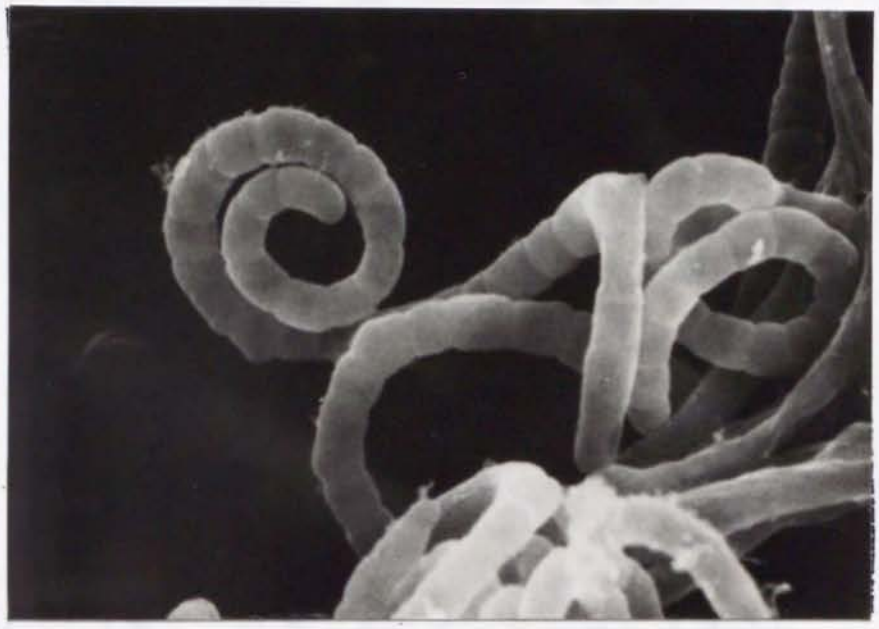
Chapter II Taxonomy, Fermentation, Isolation and Physico-chemical Properties

II-1 Taxonomic studies on the producing organism

The producing organism, strain No. 1358, was isolated from a soil sample collected at Tochigi City, Tochigi Prefecture, Japan.

Morphological observations were made with a light and a scanning electron microscope (Fig. 1) on cultures grown at 30 ℃ for 2 to 3 weeks on yeast extract - malt extract agar, oatmeal agar and inorganic salts - starch agar. The substrate mycelia were well developed and branched without fragmentation. The aerial mycelia branched monopodially, and formed spiral, sometimes looped, spore chains with 10 to 50 spore per chain. The spores had a smooth surface and were subglobose to oblong in shape with a size of 0.5 to 0.7 by 0.5 to 0.9 μm. These spore masses often appeared to coalesce in moist globules. Sclerotic granules, sporangia and zoospores were not observed. The cultural characteristics of strain No. 1358 are summarized in Table 1. The aerial mass color was brownish grey to greyish brown. Reverse side of growth was yellowish white to greyish yellow. Melanoid and other soluble pigments were not produced. The physiological characteristics and utilization of carbon sources of strain No. 1358 are summarized in Table 2 and 3. Hydrolized whole

Fig. 1. Scanning electron microphotography of aerial mycelia of strain No. 1358.



The bar represents 1 μm.

Table 1. Cultural characteristics of strain No. 1358.

Medium	Growth	Aerial mycelium	Reverse side of substrate mycelium	Soluble pigments
Yeast extract-malt extract agar	Abundant	White, brownish gray (6D2), grayish brown (7F3)	Grayish yellow (4B3)	None
Oatmeal agar	Moderate	Grayish brown (5F3)	Yellowish white (3A2)	None
Inorganic salts-starch agar	Abundant	Brownish gray (7D-E2)	Grayish yellow (4B3)	None
Glycerol-asparagine agar	Abundant	Brownish gray (6C2)	Yellowish white (4A2)	None
Peptone-yeast extract-iron agar	Moderate	None	Light yellow (4A4-5)	None
Tyrosine agar	Abundant	Orange gray (5B2), brownish gray (7E2)	Grayish orange (5B3)	Scant, grayish orange
Glucose-asparagine agar	Abundant	Brownish gray (6C-D2), grayish brown (7F3)	Yellowish white (4A2)	None
Nutrient agar	Moderate	None	Pale yellow (3A3)	None
BENNETT agar	Abundant	Dark brown (6F4)	Pale yellow (3A3)	None
Sucrose-nitrate agar	Abundant	None	Pale yellow (3A3)	None

Table 2. Physiological characteristics of strain No. 1358.

Temperature range for growth	16～33°C
Optimum temperature for growth	26～32°C
Liquefaction of gelatin	Weakly positive
Coagulation of milk	Negative
Peptonization of milk	Weakly positive
Hydrolysis of starch	Positive
Melanoid production	Negative
Decomposition of cellulose	Negative
Nitrate reduction	Positive
NaCl tolerance	0～2%

Table 3. Utilization of carbon sources by strain No. 1358.

Carbon source	Utilization
Glucose	+
L-Arabinose	+
D-Xylose	+
Inositol	+
Mannitol	+
D-Fructose	+
L-Rhamnose	+
Sucrose	+
Raffinose	+
No addition	−

−: No growth, +: good growth.

Table 4. Differences between strain No. 1358 and *Streptomyces parvulus* IFO 13193.

	No. 1358	IFO 13193
Temperature range for growth (°C)	16～33	14～40
NaCl tolerance (%)	2	10

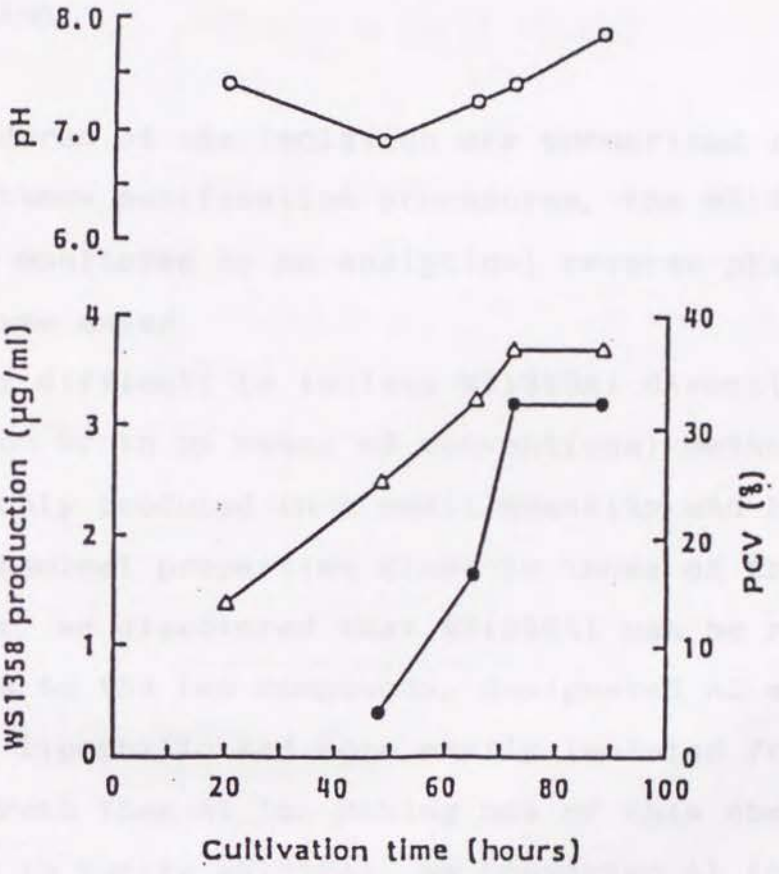
cell of strain No. 1358 contained the LL-diaminopimelic acid. Accordingly, the cell wall of this strain is believed to be of type I.

Based on the taxonomic properties described above, strain No. 1358 is considered to belong to the genus *Streptomyces*[1] and to be a strain of the grey series of the Pridham and Tresner grouping[2]. Strain No. 1358 was compared with *Streptomyces* specis described in literatures[3-7]. As a result, it was found that the strain proved to closely resemble *Streptomyces parvullus* IFO 13193 in detail. There, it was found that the properties of both strains were almost identical except a few differences. Table 4 shows the differences between two strains. These differences are not sufficient to consider that strain No. 1358 belongs to a distinct species. So, it is considered to be proper that strain No. 1358 is a sub-species strain of *S. parvullus*. Therefore, this strain was designated as *S. parvullus subsp. tochigiensis* No. 1358. The strain has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan under the accession No. FERM BP-1638.

II-2 Fermentation

A typical fermentation profile for the production of WS1358 is shown in Fig. 2. The production of active compounds during the fermentation was monitored by measuring the inhi-

Fig. 2. A typical time course of fermentation by strain No. 1358.



● Potency, ○ pH, △ packed cell volume (PCV).

bitory activity against DHP and assessed from the standard curve of the purified WS1358A1. The production as well as the cell growth was initiated on day 2 and rapidly reached a maximum of about 3.7 μg/ml on day 3.

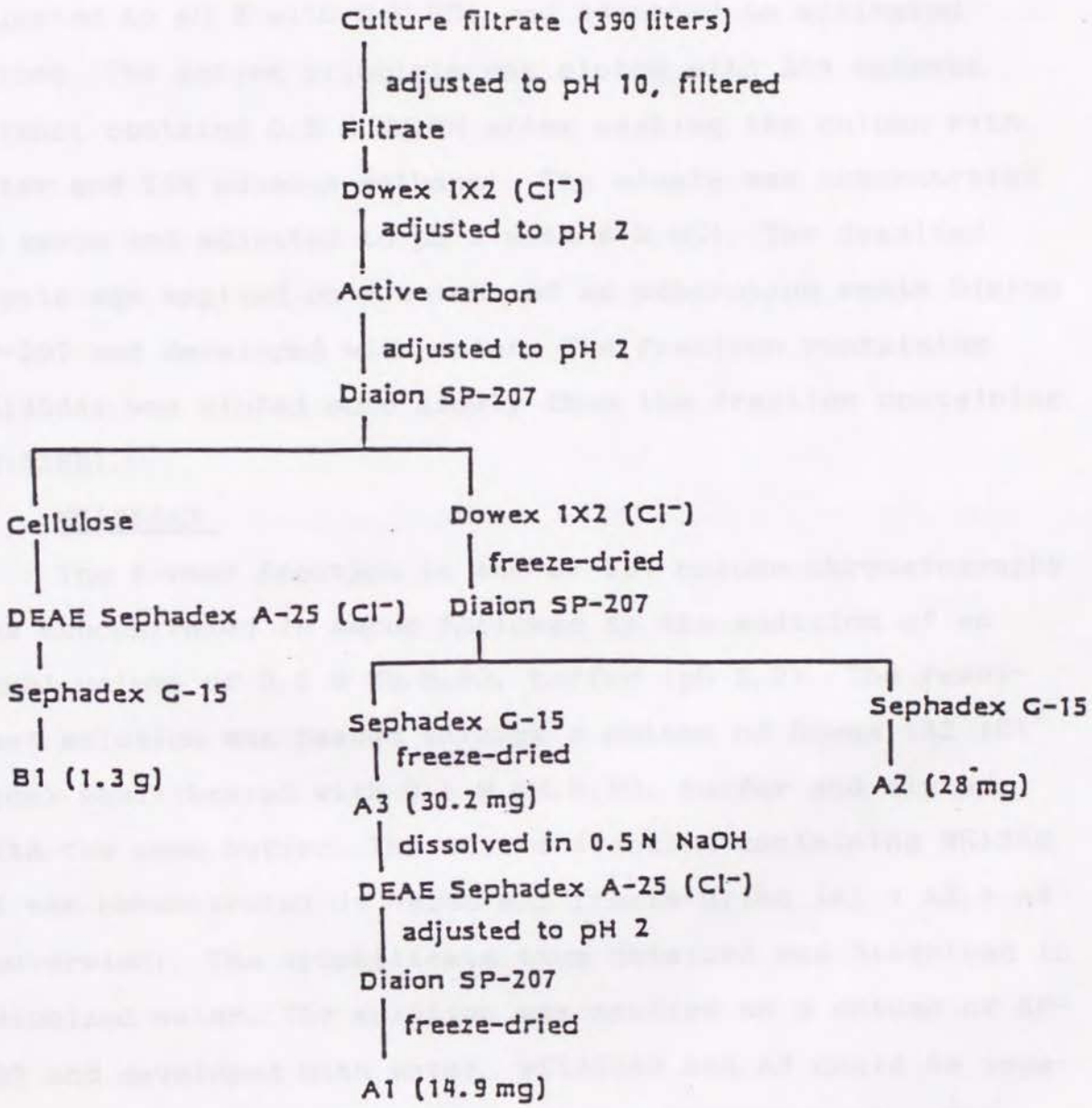
II-3 Isolation

The procedures of the isolation are summarized in Fig. 3. Throughout these purification procedures, the WS1358 compounds were monitored by an analytical reverse-phase HPLC instead of enzyme assay.

It is very difficult to isolate WS1358A1 directly from the fermentation broth by means of conventional methods, because it is only produced in a small quantity and has the same physico-chemical properties close to those of the impurities. However, we discovered that WS1358A1 can be reversibly transformed to the two compounds, designated A2 and A3, which are more lipophilic and more easily isolated from the fermentation broth than A1 is. Making use of this chemical transformation to purify WS1358A1, we converted A1 to A2 and A3 in the course of purification and finally converted the purified A3 to A1. More detailed mechanism of this chemical transformation will be described in the chapter V.

The cultured broth was filtered with the aid of diatomaceous earth. The filtrate (390 liters) was adjusted to pH 10 with 6 N NaOH and allowed to stand for 2 hours at room

Fig. 3. Isolation procedure of WS1358 compounds.



temperature (A2, A3 → A1 conversion). The resultant precipitate was filtered and discarded. The filtrate was passed through a column of Dowex 1X2 (Cl^- type). The active principle was eluted with 0.2 M NaCl solution after washing the column with water and 0.1 M NaCl solution. The eluate was adjusted to pH 2 with 6 N HCl and adsorbed to activated carbon. The active principle was eluted with 25% aqueous methnol containg 0.5 N NH_4OH after washing the column with water and 25% aqueous methanol. The eluate was concentrated *in vacuo* and adjusted to pH 2 with 6 N HCl. The desalted eluate was applied on a column of an adsorption resin Diaion SP-207 and developed with water. The fraction containing WS1358A1 was eluted more slowly than the fraction containing WS1358B1.

WS1358A3

The former fraction in the SP-207 column chromatography was concentrated *in vacuo* followed by the addition of an equal volume of 0.2 M $NH_4H_2PO_4$ buffer (pH 2.3). The resultant solution was passed through a column of Dowex 1X2 (Cl^- type) equilibrated with 0.1 M $NH_4H_2PO_4$ buffer and eluted with the same buffer. The active fraction containing WS1358 A1 was concentrated *in vacuo* and freeze-dried (A1 → A2 + A3 conversion). The lyophilizate thus obtained was dissolved in deionized water. The solution was applied on a column of SP-207 and developed with water. WS1358A2 and A3 could be separated from each other in this column chromatography. The fraction containg WS1358A3 was neutralized with 6 N NaOH and

applied on a column of Sephadex G-15. The eluate was freeze-dried to give a white powder of WS1358A3 (35.4 mg).

WS1358A1

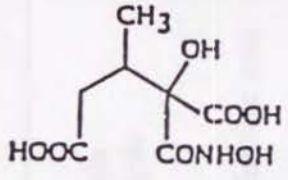
WS1358A3 (30.2 mg) was dissolved in 0.5 N NaOH (3.5 ml) and allowed to stand for 30 minutes at room temperature (A3→A1 conversion), then diluted with water after neutralization with 6 N HCl. The resultant solution was applied on a column of DEAE-Sephadex A-25 (Cl^- type) and eluted with water, 0.1 M NaCl and 0.2 M NaCl solution. The active fraction was adjusted to pH 2 with 6 N HCl and concentrated *in vacou*, then applied on a column of SP-207 and developed with water. The eluate was neutralized with 1 N NaOH and freeze-dried to give a white powder of WS1358A1 (14.9 mg).

WS1358B1

The fraction containing WS1358B1 obtained by the 1st SP-207 column chromatography was neutralized with 1 N NaOH and concentrated *in vacuo*. The resultant solution was applied on a column of Cellulose CF11 and washed with 2-propanol, then developed with 75% aqueous 2-propanol. The active fraction was concentrated *in vacuo* and applied on a column of DEAE-Sephadex A-25 (Cl^- type). The active fraction was eluted with 0.2 M NaCl solution and concentrated *in vacuo*. The eluate was applied on a column of Sephadex G-15 equilibrated with water, then eluted with water. The desalted eluate was concentrated *in vacuo* to give the residue. The residue was recrystallized from aqueous methanol to give a colorless powder of WS1358B1 (1.3 g).

II-4 Physico-chemical properties

Physico-chemical properties and spectral data of WS1358A1 and B1 Na salts are summarized in Table 5. The two compounds are soluble in water, slightly soluble in methanol and insoluble in acetone, ethy acetate, chloroform and *n*-hexane. They give positive color reactions for ferric chloride, Ehrlich, ninhydrin and iodine vapor reagents, but not for Dragendorff, Molish and cerium sulfate reagents. These data suggested that the compounds have *N*-hydroxyl group. The IR, ^{1}H-NMR and ^{13}C-NMR spectra are shown in Fig. 4, 5, 6, respectively. The structures of WS1358 A1 and B1 were deduced as shown below on the basis of the physico-chemical and spectral data. Details of the structural elucidation will be described in the next chapter.



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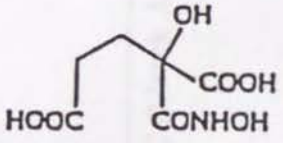


WS1358B1

Table 5. Physico-chemical properties of WS1358A1 and B1.

	A1	B1
MP (°C, dec)	98～100	92～93
Molecular formula	$C_7H_9NO_7Na_2$	$C_6H_7NO_7Na_2$
Elementary analysis	Calcd for $C_7H_9NO_7Na_2 \cdot H_2O$	Calcd for $C_6H_7NO_7Na_2 \cdot H_2O$
Calcd:	C 29.69, H 3.92, N 4.95, Na 16.24	C 26.78, H 3.37, N 5.20, Na 17.08
Found:	C 30.05, H 3.84, N 4.88, Na 15.89	C 26.44, H 3.28, N 5.10, Na 16.88
FAB-MS (*m/z*) for free acid	222 ($M^+ + 1$)	208 ($M^+ + 1$)
$[\alpha]_D^{23}$ (H_2O)	−14.0° (*c* 0.9)	+2.5° (*c* 1.0)
UV $\lambda_{max}^{H_2O}$ nm (ε)	End absorption	End absorption
IR ν_{max} (KBr) cm^{-1}	3500～2500, 1660, 1580, 1360, 1160, 1100, 1000, 880, 800	3500～2500, 1660, 1620, 1580, 1400, 1380, 1200, 1130, 1100, 880, 800
Rf value[a] (I)	0.43	0.40
(II)	0.56	0.46

[a] Silica gel TLC (Merck Art. No. 5715), solvent (I) BuOH - AcOH - H_2O (2:1:1), (II) 2-PrOH - H_2O (65:35).

Fig. 4. IR spectra of WS1358A1 and B1 (KBr).

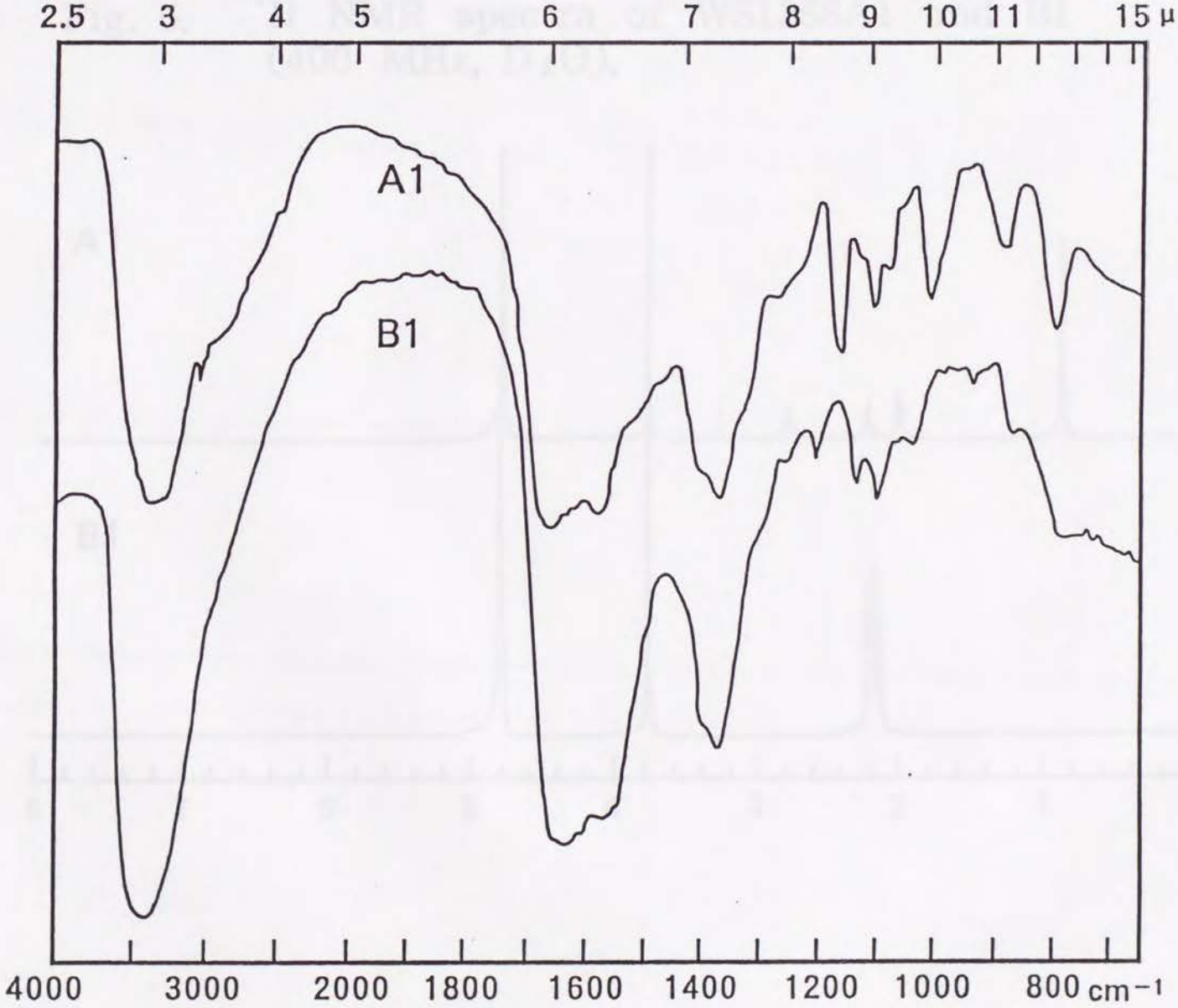


Fig. 5. ^{1}H NMR spectra of WS1358A1 and B1 (400 MHz, D_2O).

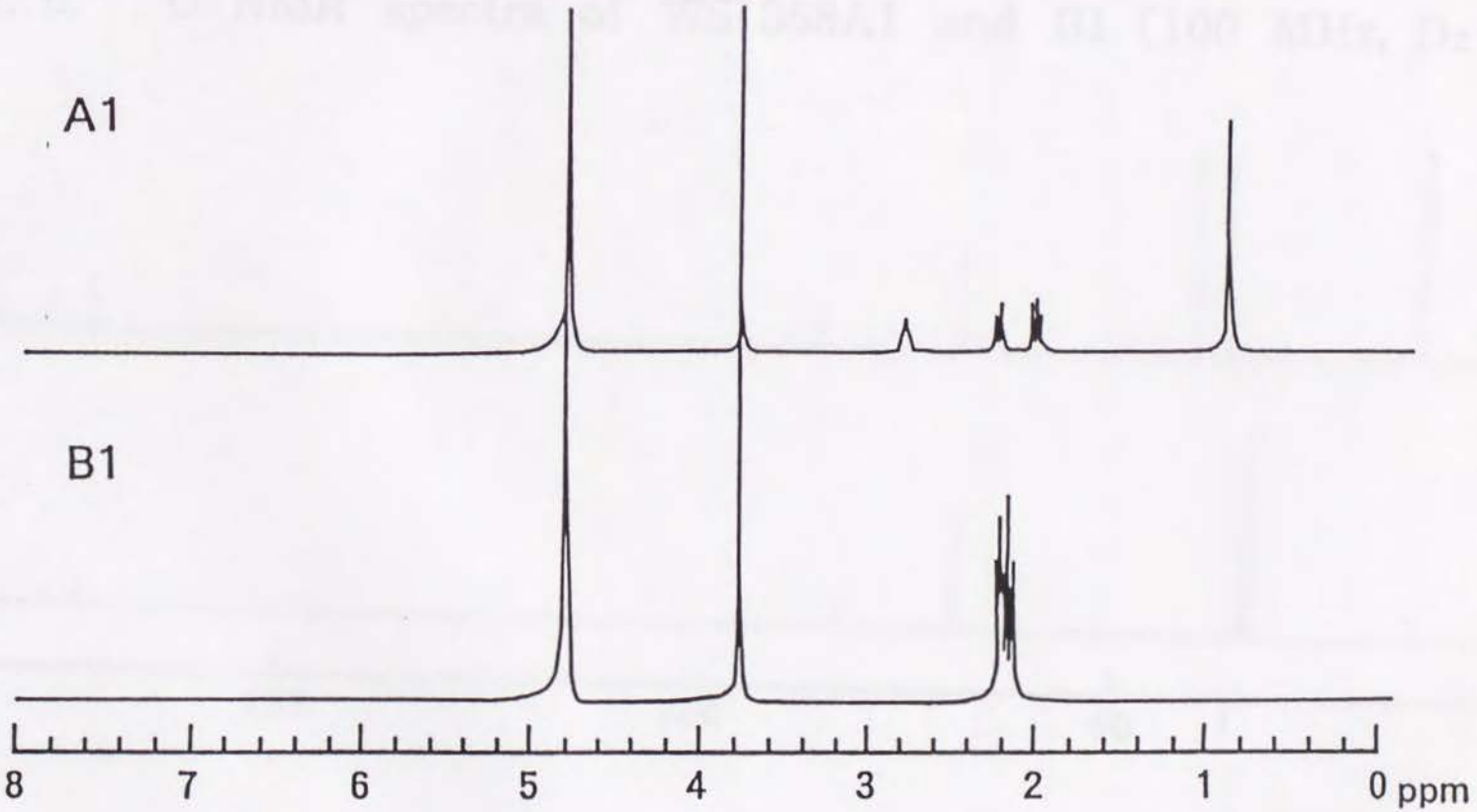
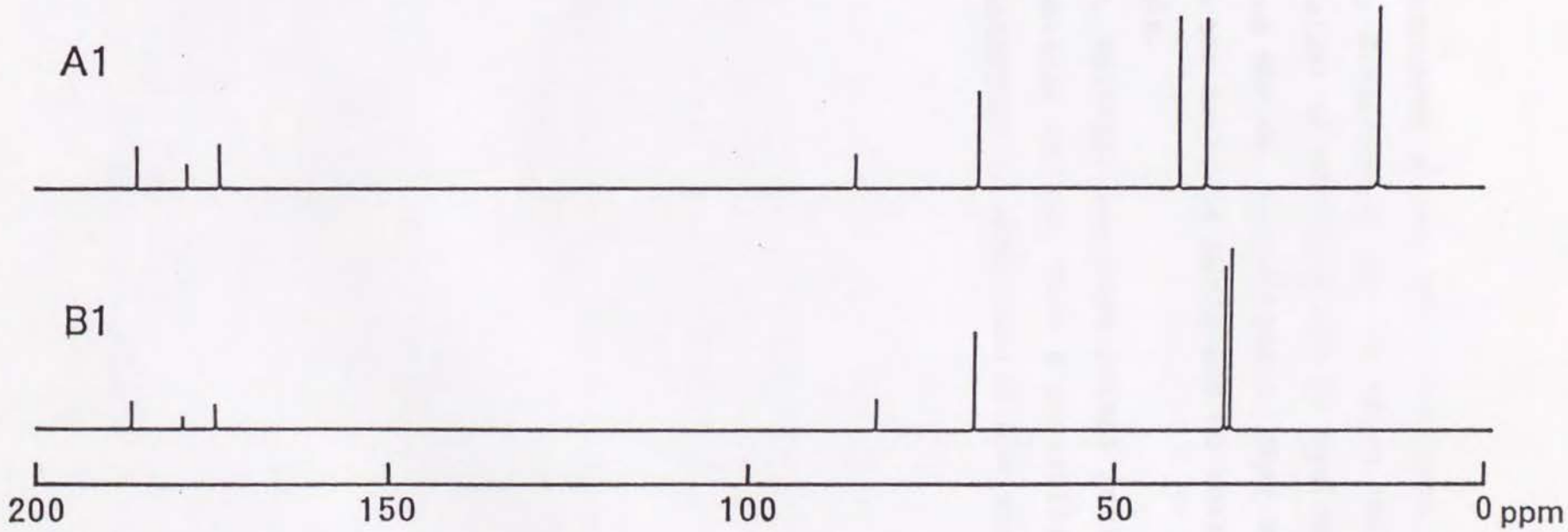


Fig. 6. ^{13}C NMR spectra of WS1358A1 and B1 (100 MHz, D_2O).



II-5 Summary

The author discovered potent DHP inhibitors, WS1358A1 and B1, produced by *Streptomyces* sp. *In vitro* inhibitory activities (IC_{50} value) of WS1358A1 and B1 against porcine renal DHP were 3 and 600 nM, respectively. They also showed no cytotoxicity to the cultured cells and no toxicity to experimental animals.

In particular, WS1358A1 has more potent inhibitory activity than cilastatin and may have a possibility to be developed as a counterpart in combination antimicrobial.

Experimental

Taxonomic studies

The media and procedures used for cultural and physiological characterization of strain No. 1358 were described by Shirling and Gottlieb[8]. Each culture was incubated at 30℃ for 2 to 3 weeks before observation. The temperature range for growth was determined on yeast extract - malt extract agar using a temperature gradient incubator (Advantec Toyo Co.). The color names used in these studies were based on the Methuen Handbook of Colour[9]. The chemical composition of the cell wall was analyzed by the methods described by Becker *et al.*[10], and Lechevalier and Lechevalier [11]. Utilization of carbon sources was examined according to the method of Pridham and Gottlieb[12].

Fermentation

A loopful of the slant culture of strain No. 1358 was inoculated to a 500 ml flask containig 160 ml of the seed medium composed of corn starch 1.0%, glycerol 1.0%, glucose 0.5%, cotton-seed flour 1.0%, dried yeast 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% (pH 6.5). The flasks were shaken on a rotary shaker (220 rpm) for 3 days at 30℃. A 200-liter jar fermentor containing 150 liters of production medium composed of glucose 1.0%, glycerol 2.0%, cotton-seed flour 1.0%, soy bean meal 1.0%, dried yeast 0.5% and $CaCO_3$ 0.3% (pH 7.0) was inoculated with 3 liters of the seed broth and cultured for 4 days at 30℃, aerated at 150 liters per minute and agitated at 250 rpm.

Enzyme assay

The prodution and initial purification of WS1358 compounds were monitored with the enzyme assay to determine the inhibitory activity. Partially purified porcine renal DHP is able to hydrolyze the unsaturated dipeptide, glycyldehydrophenylalanine (GDP) as substrate[13]. The activity of the enzyme was determined by observing the decline in absor-

bance at 275 nm. Inhibitory effect of a compound on DHP activity was also measured in a method shown in Fig. 7. Inhibition percent, I %, was calculated as follows, I % = (E - T)/E X 100, where E was DHP activity without the compound, T was DHP activity with the compound.

Materials

Partial purification of DHP from porcine kidney of was carried out using procedures slightly different from previously reported method[14]. Details were described in the chapter IV. GDP was synthesized by the method previously described[13]. Cilastatin was purified from a comercially available drug, Zienam.

Fig. 7. Assay method of dehydropeptidase activity.

Sample or H_2O (0.025 ml)

- 0.2 ml of 0.05 M MOPS buffer (pH 7.0)
- 0.025 ml of DHP solution

Preincubation at 37℃ for 5 minutes

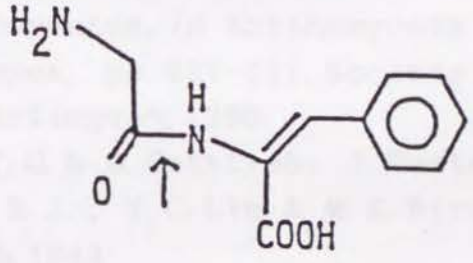
- 1 ml of 0.1 mM GDP in 0.05 M MOPS buffer

Incubation at 37℃ for 30 minutes

- 1 ml of 0.1 M EDTA

Measurement of absorbance at 275 nm

Inhibition (%) = (E-T)/E X 100
E; DHP activity with H_2O
T; DHP activity with sample



Dehydropeptide

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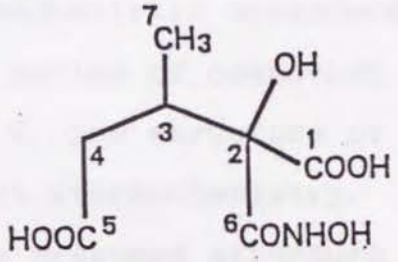
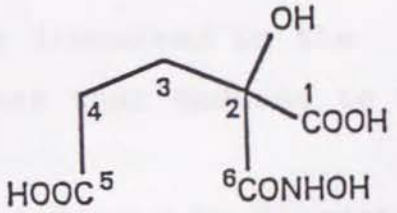
Chapter III Structure Determination of WS1358A1 and WS1358B1

III-1 Determination of plane structure

WS1358A1 (1) was isolated as a colorless powder from the fermentation broth of *Streptomyces* sp. No. 1358: Na salt, $C_7H_9NO_7Na_2$ (elemental analysis); free acid, $C_7H_{11}NO_7$ (fast atom bombardment (FAB)-MS); $(\alpha)_D$ -14.0° (*c* 0.9, H_2O, Na salt). From the same culture broth, WS1358B1 (2) was isolated as a colorless powder: Na salt, $C_6H_7NO_7Na_2$ (elemental analysis); free acid, $C_6H_9NO_7$ (FAB-MS); $(\alpha)_D$ +2.5° (*c* 1.0, H_2O, Na salt).



1



2

The ^{13}C NMR spectrum of 2 (D_2O, pD 7.0) revealed all the carbon signals, which were assignable to three carbonyls (δ 185.3 (s, C-5), 178.2 (s, C-1), 173.6 (s, C-6)), one tertiary alcohol (δ 82.7 (s, C-2)), and two methylenes (δ 35.6 (t, C-3), 34.8 (t, C-4)). The proton signals corresponding to the two methylenes were observed at δ 2.25-2.11

(4H) as multiplet in the ^{1}H NMR spectrum of 2 (D_2O, pD 7.0). The linkage of all the six carbons in 2 was determined by using a 2D INADEQUATE technique[1], revealing the carbon framework of 2 as depicted in Fig. 1.

The remaining problem is to elucidate the position of the function containing nitrogen. As described in the preceding chapter, 2 showed a positive color reaction (purple) against ferric chloride, suggesting the presence of a hydroxamic acid function in 2. In the ^{13}C NMR spectrum, the carbonyl of a hydroxamic acid usually resonates at higher-field region than that of the corresponding carboxylic acid*. Therefore, the C-6 signal at δ 173.6 (the highest-field signal in the carbonyl region) was assigned to the hydroxamic carbonyl carbon. This was also corroborated by the mechanistic consideration in an alternative synthesis of this series of compounds as will be discussed in the chapter V. The structure of WS1358B1 was thus deduced to be 2 without stereochemistry.

The presumed structure of 2 was confirmed by a synthesis as follows (Fig. 2). Compound 3, prepared by Michael condensation of methyl acrylate and dimethyl malonate, was

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* This was studied using model compounds: in the ^{13}C NMR spectra (CD_3OD), the carbonyl of 3-phenylpropionic acid resonated at δ 176.7, while conversion to the corresponding hydroxamic acid shifted the resonance of the carbonyl up-field to δ 171.9.

Fig. 1. The partial structure of **2** and its ^{13}C NMR data (chemical shifts in ppm).

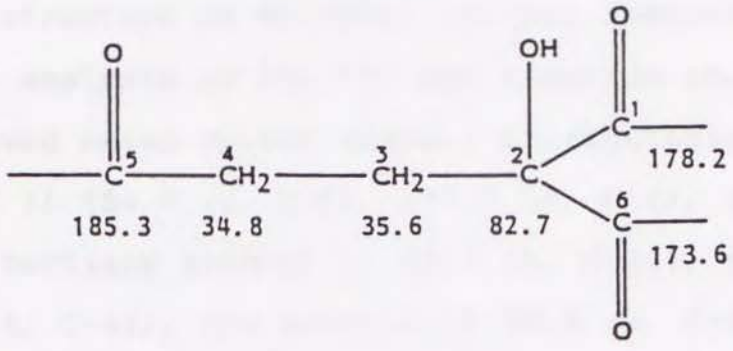
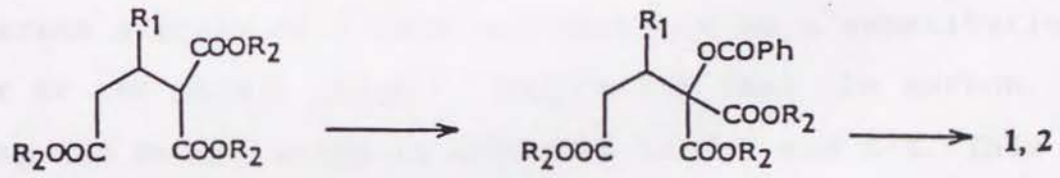


Fig. 2. Synthesis of WS1358A1 and B1 racemates.



3 $R_1=H$ $R_2=CH_3$
5 $R_1=CH_3$ $R_2=Et$

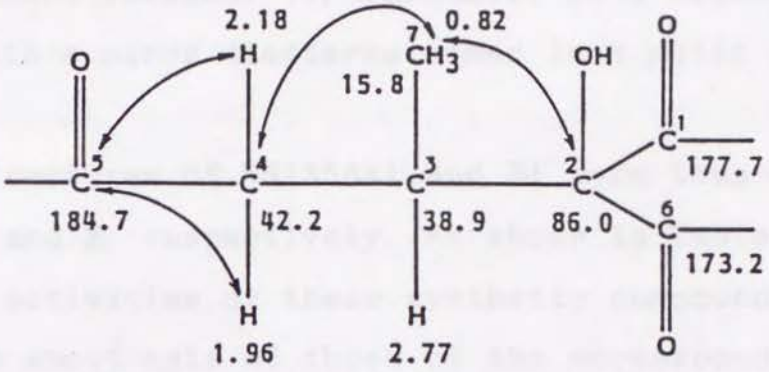
4 $R_1=H$ $R_2=CH_3$
6 $R_1=CH_3$ $R_2=Et$

subjected to oxidation with benzoyl peroxide to give compound 4. Treatment of 4 with hydroxylamine (2 equiv), followed by a sequence of purification processes described in the preceding paper provided B1 (2, racemate) as the Na salt.

The structure of WS1358A1 (1) was deduced in a similar manner by analysis of its ^{13}C NMR spectrum (D_2O, pD 7.0), which showed seven carbon signals attributable to three carbonyls (δ 184.7 (s, C-5), 177.7 (s, C-1), 173.2 (s, C-6)), one tertiary alcohol (δ 86.0 (s, C-2)), one methylene (δ 42.2 (t, C-4)), one methine (δ 38.9 (d, C-3)), and one methyl (δ 15.8 (q, C-7)). The methyl proton signal was observed at δ 0.82 (3H, d, J = 6.5 Hz) and the methylene and methine signals appeared at δ 2.18 (1H, dd, J = 13.5 and 3 Hz), 1.96 (1H, dd, J = 13.5 and 11 Hz) and at δ 2.77 (1H, m) in the ^1H NMR spectrum (D_2O, pD7.0) of 1.

The carbon skeleton of A1 (1) was deduced to be the one depicted in Fig. 3 by the following NMR studies. The downfield shifts observed for C-2, C-3 and C-4 (3.3, 3.3 and 7.4 ppm, respectively) of 1 as compared with the corresponding carbon signals of 2 were attributable to a substitution effect of the methyl group[2], indicating that the carbon bearing the methyl group is adjacent to C-2 and C-4. This was corroborated by a COLOC experiment[3], which showed that the methyl protons are coupled to C-2 and C-4, and further both methylene protons of the C-4 are coupled to the C-5 carbonyl. These data supported the carbon framework depicted

Fig. 3. The partial structure of 1 and its NMR data (chemical shifts in ppm and ^{1}H-^{13}C relationships).



in Fig. 3. As in the case of WS1358B1, the highest-field signal (C-6, δ 173.2) of the three carbonyls was assignable to the hydroxamic acid and, consequently, the structure of A1 was proposed to be 1 without stereochemistry.

The presumed structure of 1 was ascertained by a sequence of synthetic reactions similar to that used for B1 (Fig. 2). Michael condensation of ethyl crotonate and diethyl malonate gave compound 5, which was similarly subjected to oxidation with benzoyl peroxide to yield benzonate 6. Treatment of 6 with hydroxylamine and subsequent purification afforded WS1358A1 (1, racemate) as a major product together with a minor diastereoisomer in a ratio of about 3:2.

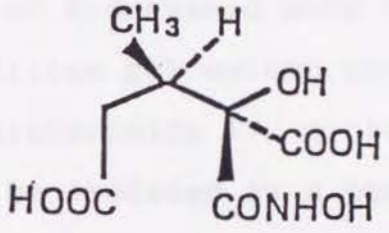
The structures of WS1358A1 and B1 were thus established as being 1 and 2, respectively. As shown in Table 1, the biological activities of these synthetic compounds were found to be about half of those of the corresponding natural products, respectively.

Table 1. IC_{50} of synthetic and natural WS1358 compounds against porcine DHP.

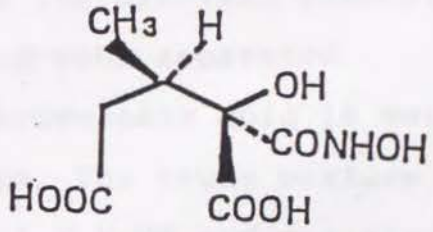
Compound	IC_{50} (ng/ml)	
	Synthetic (DL)	Natural
A1 (1)	1.5	0.83
B1 (2)	340	150

III-2 Determination of absolute structure

WS1358A1 (1) is a new, potent dehydropeptidase (DHP) inhibitor isolated from a *Streptomyces*. In the previous section, we described the structure elucidation and the synthesis (as a racemate form) of this substance. The stereochemistry of this substance has remained unsolved, however. In this section, the author will describe an asymmetric synthesis of WS1358A1, which leads to a conclusion that the natural product possesses the R absolute configuration at C-3, although the configuration at C-2 still remains undetermined. WS1358A1 exists as a mixture of the diastereoisomers 1a and 1b at C-2 probably through compounds 7 and 8. It is unclear which isomer is stable and predominant in this mixture.

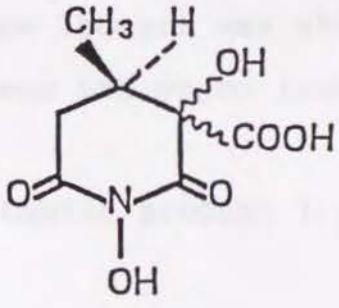


a

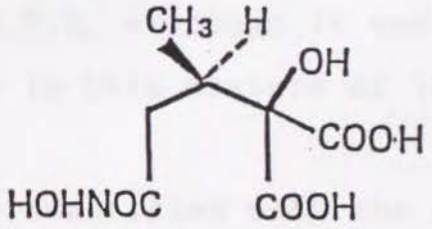


b

1



7

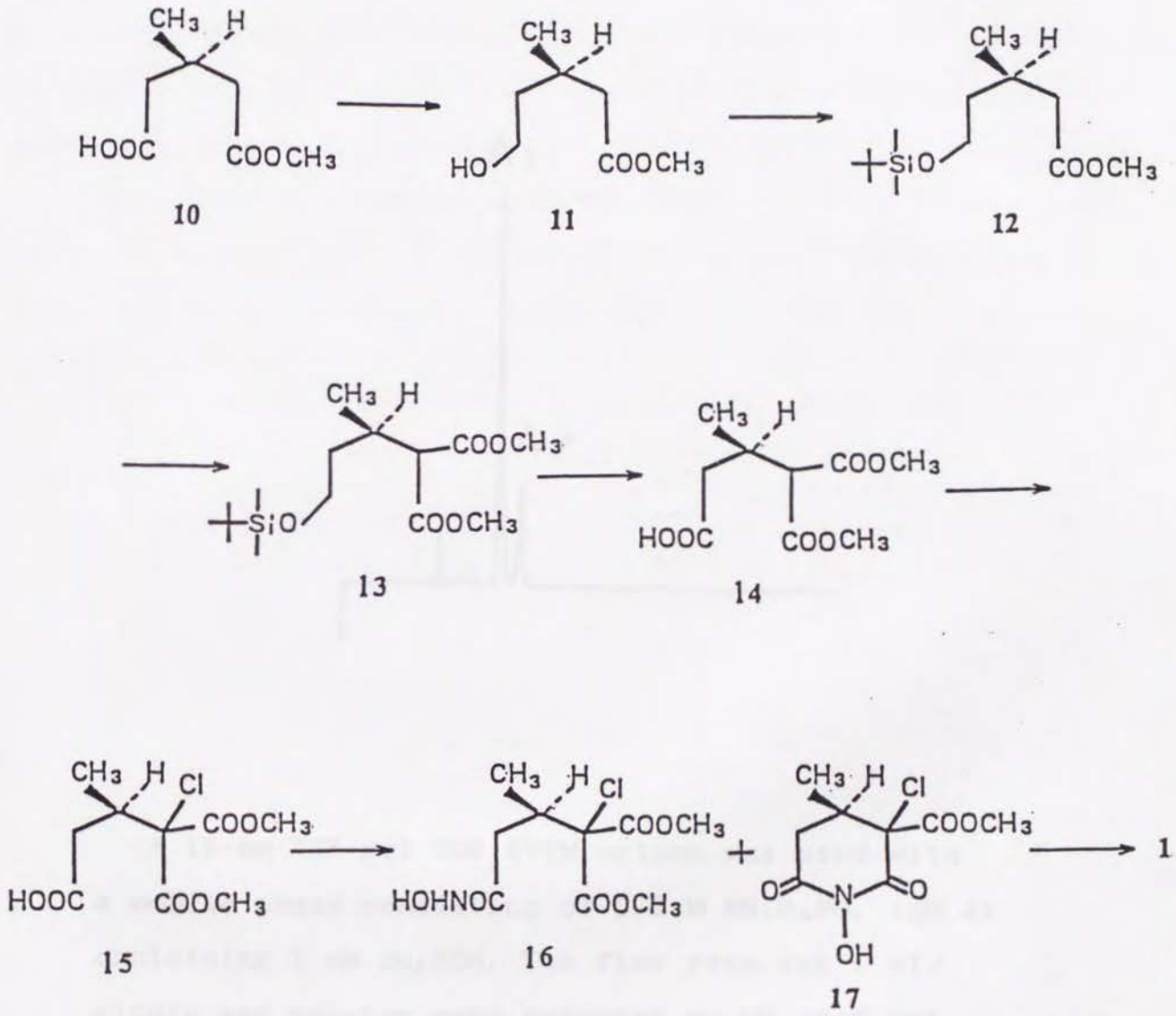


8

As a starting material for the synthesis (Fig. 4) of the optically active WS1358A1, we chose the half ester of β-methylglutaric acid. The R-(+) enantiomer 10 of this half ester was obtained by resolution using cinchonimide according to the method described in the literature[4]. Reduction of 10 using diborane gave alcohol 11 in 94.3% yield, which, after protection with the t-butyl-dimethyl silyl group (12, 77.6%), was allowed to react with dimethyl carbonate in the presence of lithium diisopropylamide to afford malonate 13 in 45.6% yield. Jones oxidation of this product yielded carboxylic acid 14 (66.7%), which was then subjected to chlorination with hypochlorite to provide chloride 15 in 68.0% yield. The carboxylic acid function in 15 was next led to the active ester by using *N*-hydroxysuccinimide and then allowed to react with hydroxylamine to yield a mixture of hydroxamic acid 16 and its cyclized product 17 (57.5%). Silica gel column chromatography separated *N*-hydroxyglutarimide 17, although hydroxamic acid 16 was unable to be isolated as a pure form. The crude mixture of 16 and 17 was finally treated with 1 N NaOH and purified by column chromatography on Diaion SP-207 to provide WS1358A1 in 55.8% yield. The product was found to be amixture of the diastereoisomers 1a and 1b as shown on HPLC (Fig. 5). The ratio of these isomers was about 7:2, although it was ambiguous which was the major isomer in this mixture of 1a and 1b.

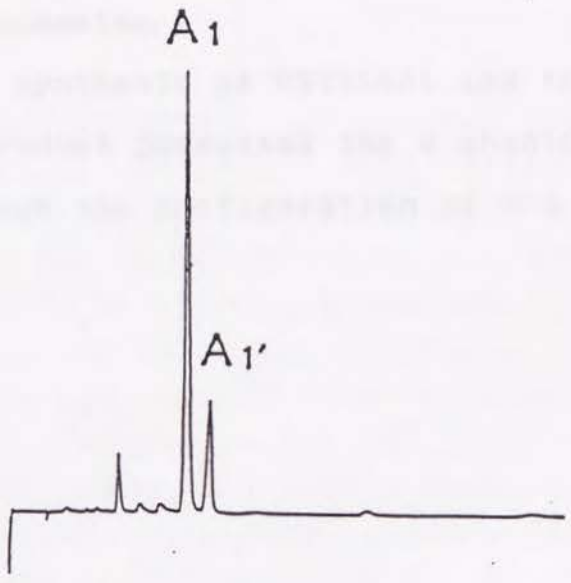
The synthetic product 1 was identified with the natural

Fig. 4. Asymmetric synthesis of WS1358A1.



product on HPLC. The inhibitory activity of this synthetic product against renal DHP was 0.88 ng/ml in IC_{50} (the natural product, 0.61 ng/ml).

Fig. 5. HPLC analysis of asymmetrically synthesized WS1358A1



A 15-cm TSK gel ODS 80TM column was used with a mobile phase consisting of 0.2 M $NH_4H_2PO_4$ (pH 4) containing 1 mM Bu_4NOH. The flow rate was 1 ml/minute and solutes were detected by UV (210 nm). The retention times of A1 and A1' were 7.15 and 8.13 minutes, respectively.

III-3 Summary

The structures of WS1358A1 and B1, new dehydropeptidase inhibitors isolated from *Streptomyces parvulus* subsp. *tochigiensis* No. 1358, have been established to be 2-hydroxy-2-hydroxyaminocarbonyl-3-methylglutaric acid and 2-hydroxy-2-hydroxyaminocarbonylglutaric acid (2), respectively, without streochemistry on the basis of spectroscopic evidence and synthesis of the racemates.

The aymmmetric synthesis of WS1358A1 led to a conclusion that the natural product possesses the R absolute configuration at C-3, although the configuration at C-2 still remains undetermined.

Experimental

General

IR spectra were recorded on a Jasco A-102 spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AM400 spectrometer. MS were determined with a VG ZAB-SE spectrometer. Optical rotations were measured on a Jasco DIP-400 polarimeter. Analytical HPLC was performed on a Hitachi 655 liquid chromatograph using a YMC ODS-5 column (4.6 X 250 mm) and preparative HPLC was carried out using a YMC ODS-5 column (20 X 250 mm).

WS1358A1 (1)

For isolation of WS1358A1, see chapter II: Na salt, $(\alpha)_D$ -14.0° (c 0.9, H_2O); IR (KBr) cm^{-1} 1660, 1580; ^{1}H NMR (D_2O) δ 2.77 (1H, m), 2.18 (1H, dd, J = 13.5 and 3 Hz), 1.96 (1H, dd, J = 13.5 and 11 Hz), 0.82 (3H, d, J = 6.5 Hz); ^{13}C NMR (D_2O) δ 184.7 (s), 177.7 (s), 173.2 (s), 86.0 (s), 42.2 (t), 38.9 (d), 15.8 (q); *Anal* Calcd. for $C_7H_9NO_7Na_2 \cdot H_2O$; C 29.69, H 3.92, N 4.95, Na 16.24; Found, C 30.05, H 3.84, N 4.84, Na 15.89. Free acid, FAB-MS *m/z* 222 (M^++1); ^{1}H NMR (D_2O-H_2O) δ 2.89 (1H, m), 2.39 (1H, dd, J = 15 and 3.5 Hz), 2.23 (1H, dd, J = 15 and 10 Hz), 0.95 (3H, d, J = 6.5 Hz); ^{13}C NMR (D_2O-H_2O) δ 182.2 (s), 177.4 (s), 173.0 (s), 85.8 (s), 40.5 (t), 38.5 (d), 15.9 (q).

WS1358B1 (2)

For isolation of WS1358B1, see chapter II: Na salt, $(\alpha)_D$ +2.5° (c 1.0, H_2O); IR (KBr) cm^{-1} 1660, 1620, 1580; ^{1}H NMR (D_2O) δ 2.25-2.11 (4H, m); ^{13}C NMR (D_2O) δ 185.3 (s), 178.2 (s), 173.6 (s), 82.7 (s), 35.6 (t), 34.8 (t); *Anal* Calcd for $C_6H_7NO_7Na_2 \cdot H_2O$; C 26.78, H 3.37, N 5.20, Na 17.08; Found, C 26.44, H 3.28, N 5.10, Na 16.88. Free acid, FAB-MS *m/z* 208 (M^++1); ^{13}C NMR (D_2O) δ 180.3 (s), 175.9 (s), 171.4 (s), 81.4 (s), 34.0 (t), 31.2 (t).

Dimethyl 2-(Methoxycarbonyl)glutarate (3)

A solution of dimethyl malonate (13.2 g, 0.1 mol) in benzene (30 ml) was added to a suspension of sodium hydride (60% dispersion in mineral oil, 400 mg, 0.01 mol) in benzene (50 ml) at 0℃ under an atmosphere of nitrogen. To this mixture a solution of methyl acrylate (8.6 g, 0.1 mol) in benzene (30 ml) was added and the mixture was stirred at 80℃ for 1 hour. The reaction mixture was cooled to room temperature poured into ice water, and extracted with ether. The extract was washed with water, dried over $MgSO_4$ and concentrated to give 3 as an oil (19.4 g): electron impact (EI)-MS, *m/z* 218 (M^+); ^1H NMR ($CDCl_3$) δ 3.73 (6H, s), 3.69 (3H, s), 3.51 (1H, t, J = 7 Hz), 2.5-2.2 (4H, m).

Dimethyl 2-Benzoyloxy-2-(methoxycarbonyl)glutarate (4)

A solution of 3 (2.18 g, 10 mmol) in benzene (30 ml) was added to a suspension of sodium hydride (60% dispersion in mineral oil, 400 mg, 10 mmol) in benzene (50 ml) under an atmosphere of nitrogen. The mixture was refluxed for 1 hour, during which the mixture became clear, and then cooled to room temperature. To this mixture a solution of benzoyl peroxide (1.82 g, 7.5 mmol) in benzene(30 ml) was added dropwise under ice-bath cooling and the mixture was stirred at room temperature for 15 hours. The reaction mixture was poured into ice water and extracted with ether. The extract was washed with water, dried over $MgSO_4$ and evaporated to give an oil (3.85 g), which was purified by chromatography on silica gel, eluting with 20% ethyl acetate in hexane. The fractions containing the desired product were combined and concentrated to give 4 as an oil (1.83 g): FAB-MS, *m/z* 339 (M^++1); ^1H NMR ($CDCl_3$) δ 8.09 (2H, br d, J = 8 Hz), 7.61 (1H, br t, J = 8 Hz), 7.47 (2H, br t, J = 8 Hz), 3.84 (6H, s), 3.64 (3H, s), 2.74 (2H, t, J = 7 Hz), 2.49 (2H, t, J = 7 Hz).

WS1358B1 (2, racemate)

Hydroxylamine hydrochloride (139 mg, 2 mmol) was dissolved in 50% aqueous methanol (0.8 ml) and 10 N NaOH (0.4

ml, 4 mmol) was added. To this mixture a solution of 4 (338 mg, 1 mmol) in methanol (2 ml) was added and the mixture was stirred at room temperature for 3 hours. The reaction mixture, after being acidified with 1 N HCl to pH 2, was washed with ethyl acetate. The aqueous solution was neutralized with 1 N NaOH and concentrated to remove the organic solvents. The resulting aqueous layer was passed through a column of Dowex 50WX2 (H^+, 30 ml) and eluted with water. The eluate was lyophilized to give a powder (435 mg), which was purified by preparative HPLC on YMC ODS-5 eluting with 0.1% TFA in water. The fraction containing the product corresponding to WS1358B3 (equivalent to WS1358A3) was collected and lyophilized to give a powder (60 mg), which was treated with 0.1 N NaOH (60 ml) for 2 hours and neutralized to pH 7 with Dowex 50WX2 (H^+). The solution was lyophilized to give WS1358B1 (2, racemate, 52 mg). This synthetic product was physically identical with the natural B1 in all respects except for optical rotation.

Diethyl 2-(Ethoxycarbonyl)-3-methylglutarate (5)

Compound 5 was prepared from diethyl malonate (16 g, 0.1 mmol) and ethyl crotonate (11.4 g, 0.1 mmol) in a manner similar to that for 3: yield 25.1 g (oil); EI-MS, *m/z* 274 (M^+); ^{1}H NMR ($CDCl_3$) δ 4.27-4.09 (6H, m), 3.40 (1H, d, J = 7 Hz), 2.75 (1H, m), 2.55 (1H, dd, J = 16 and 5 Hz), 2.30 (1H, dd, J = 16 and 8.5 Hz), 1.32-1.22 (9H, m), 1.08 (3H, d, J = 7 Hz).

Diethyl 2-Benzoyloxy-2-(ethoxycarbonyl)-3-methylglutarate (6)

Compound 6 was prepared from 5 (274 mg, 1 mmol) in a manner similar to that for 4: yield 250 mg; EI-MS, *m/z* 394 (M^+); ^{1}H NMR ($CDCl_3$) δ 8.08 (2H, m), 7.67-7.42 (3H, m), 4.37-4.23 (4H, m), 4.17 (2H, q, J = 7 Hz), 3.05 (1H, m), 2.81 (1H, dd, J = 16 and 3.5 Hz), 2.32 (1H, dd, J = 16 and 10 Hz) 1.34-1.23 (9H, m), 1.19 (3H, d, J = 7 Hz).

WS1358A1 (1, racemate)

Compound 1 was prepared from 6 (394 mg, 1 mmol) in a manner similar to that for 2 (racemate). The reaction mixture, after being acidified with 1 N HCl, was washed with ether. The aqueous layer was neutralized with 1 N NaOH and evaporated to remove the organic solvents. The resulting aqueous solution was lyophilized and the residue was purified by chromatography on Diaion SP-207 (20 ml), eluting with water to give a product corresponding to WS1358A3. This product was treated with 0.5 N NaOH for 2 hours and neutralized to pH 7 with Dowex 50WX2 (H^+). The solution was lyophilized to give WS1358A1 (1, racemate, Na salt, 15 mg). This synthetic product was physically identical with the natural one in all respects except for optical rotation.

On purification by the Diaion SP-207 chromatography, another product corresponding to A3' (a diastereoisomer of A3) was also obtained, which was similarly treated with 0.5 N NaOH and neutralized to pH 6.5. The solution was lyophilized to give a diastereoisomer (racemate, Na salt, 10 mg) of A1: ^{1}H NMR (D_2O) δ 2.76 (1H, m), 2.08 (1H, dd, J = 14 and 3 Hz), 1.99 (1H, dd, J = 14 and 11 Hz), 0.87 (3H, d, J = 6.5 Hz); ^{13}C NMR (D_2O) δ 184.5 (s), 177.6 (s), 173.2 (s), 86.0 (s), 41.9 (t), 38.9 (d), 16.0 (q).

Methyl hydrogen β-methylglutarate (9)

3-Methylglutaric anhydride (33.1 g, 0.259 mol) was dissolved in anhydrous methanol (10.48 ml, 0.259 mol). The solution was stirred at 80℃ for 4 hours under a nitrogen atomosphere. After cooling overnight, the solution was distilled under a reduced pressure to give methyl hydrogen β-methylglutarate (9, 37.4 g, 90%): b.p. 115-118℃/1.8 mmHg.

R-(+)-Methyl hydrogen β-methylglutarate (10)

To a solution of 9 (59.6 g, 0.37 mol) in water (590 ml) and methanol (83 ml) was gradually added cinchonidine (110 g, 0.37 mol). The solution was warmed at 80℃ for 5 minutes

and filtered. After standing at 5℃ overnight, the crystal was filtered and successively recrystalized from 4% aqueous methanol (550 ml) and water (367 ml) to give cinchonidine salt of 9 (33.8 g): $[\alpha]_D^{21}$ -86° (*c* 4.4, methanol). The cinchonidine salt of 9 (33.8 g) was dissolved in hot water (200 ml) and instantly cooled. To the cooled solution was added 2 N HCl (200 ml) at 0℃. The crystalized half ester was extracted with ether. The ether layer was washed with water, dried over $MgSO_4$ and concentrated to dryness under a reduced pressure to give R-(+)-methyl hydrogen β-methylglutarate (10, 12.35 g): $[\alpha]_D^{20}$ +0.6° (*c* 100, homogeneous).

Methyl 5-Hydroxy-(3R)-methylpentanoate (11)

To a solution of R-(+)-methyl hydrogen β-methylglutarate (10, 9.6 g, 0.06 mol) in distilled anhydrous THF (60 ml) was gradually added a solution of 1 M diborane in THF (66 ml, 0.066 mol) at -17℃ with stirring under a nitrogen atomosphere. The mixture was successively stirred at -17℃ for an hour and at room temperature for 4 hours. After being cooled to 0℃ and added water (60 ml), the reaction mixture was evaporated *in vacuo* to remove THF. The resultant aqueous solution was extracted with ethyl acetate. After being successively washed with diluted HCl and $NaHCO_3$ solution and dried over $MgSO_4$, the ethyl acetate layer was concentrated to give methyl 5-hydroxy-(3R)-methylpentanoate (11, 8.26 g, 94.3%): FAB-MS; *m/z* 147 (M^++1), $[\alpha]_D^{20}$ +2.5° (*c* 1, methanol), ^{1}H-NMR (200 MHz, $CDCl_3$); δ 3.68 (3H, s), 3.68 (2H, t, *J* = 7 Hz), 2.4 to 2.05 (3H, m), 1.70 (1H, brs, OH), 1.55 (2H, m), 0.97 (3H, d, *J* = 7 Hz).

Methyl 5-*t*-Butyldimethylsiloxy-(3R)-methylpentanoate (12)

To a solution of 11 (7 g, 48 mmol) in DMF (450 ml) was added imidazole (8.16 g, 57.6 mmol) and *t*-butyldimethylsilylchloride (8.68 g, 57.6 mmol) at 0℃ under a nitrogen atomosphere. After stirring at room temperature overnight, the solution was concentrated under a reduced pressure to

give the oily residue, which was dissolved in ether. After being washed with water and dried over $MgSO_4$, the ether solution was concentrated under a reduced pressure to give methyl 5-*t*-butyldimethylsiloxy-(3R)-methylpentanoate (12, 9.67 g, 77.6%): FAB-MS *m/z* 261 (M^++1), $[\alpha]_D^{20}$ +2.1 ° (*c* 1, $CHCl_3$), ^{1}H-NMR (200 MHz, $CDCl_3$) δ 3.65 (3H, s), 3.63 (2H, t, *J* = 7 Hz), 2.42 to 2.0 (3H, m), 1.65 to 1.32 (2H, m), 0.95 (3H, d, *J* = 7 Hz), 0.88 (6H, s), 0.05 (9H, s).

Methyl 5-*t*-Butyldimethylsiloxy-2-carbomethoxy-(3R)-methylpentanoate (13)

A solution of *n*-butyllithium in hexane (1.6 M, 15 ml, 24 mmol) was added dropwise to a solution of diisopropylamine (2.42 g, 24 mmol) in THF (100 ml) at -17℃ with stirring under a nitrogen atomosphere. After stirring at the same temperature for 15 minutes, a solution of 12 (5.2 g, 20 mmol) in THF (40 ml) was added dropwise to the mixture. After stirring at -17℃ for additional 10 minutes, dimethyl carbonate (3.6 g, 40 mmol) was added dropwise to the mixture. After standing to room temperature, the mixture was stirred for 5 hours under a nitrogen atomosphere. The reaction mixture was poured into ice-water and extracted with ether. The ether layer was washed with water, dried over $MgSO_4$, and then concentrated to dryness under a reduced pressure to give a residue (6 g). The residue was purified by column chromatography on silica gel, eluted with ethyl acetate-hexane (1:19) to afford methyl 5-*t*-butyldimethylsiloxy-2-carbomethoxy-(3R)-methylpentanoate (13, 2.9 g, 45.6%) as an oil: FAB-MS, *m/z* 319 (M^++1), $[\alpha]_D^{20}$ -4.0° (*c* 1, $CHCl_3$), ^{1}H-NMR (200 MHz, $CDCl_3$) δ 3.73 (6H, s), 3.68 (2H, m), 3.37 (1H, d, *J* = 8.5 Hz), 2.4 (1H, m), 1.74 to 1.38 (2H, m), 1.02 (3H, d, *J* = 7 Hz), 0.87 (6H, s), 0.05 (9H, s).

4,4-Dimethoxycarbonyl-(3R)-methylbutyric Acid (14)

To a solution of 13 (1.4 g, 4.4 mmol) in acetone (17 ml) was added dropwise Jone's reagent (3 ml) at 0℃ with stirring, followed by successive stirring at 0℃ for 2 hours

and at room temperature for 2 hours. The reaction mixture was poured into water and extracted with ether. The ether layer was washed with water and dried over $MgSO_4$. Removal of the solvent under a reduced pressure gave an oily residue (1.28 g), which was purified by column chromatography on silica gel, eluted with ethyl acetate-$CHCl_3$ (1:4) to afford 4,4-dimethoxycarbonyl-(3R)-methylbutyric acid (14, 640 mg, 66.7%)as an oil: FAB-MS, *m/z* 219 (M^++1), $[\alpha]_D^{25}$ -8.0° (*c* 1, methanol), ^{1}H-NMR (200 MHz, $CDCl_3$) δ 10.1 (1H, br), 3.75 (6H, s), 3.50(1H, d, *J* = 7 Hz), 2.75 (1H, m), 2.62 (1H, dd, *J* = 16 and 5 Hz), 2.40 (1H, dd, *J* = 16 and 8 Hz), 1.12 (3H, d, *J* = 7 Hz).

4-Chloro-4,4-dimethoxycarbonyl-(3R)-methylbutyric Acid (15)

To a solution of 14 (640 mg, 2.94 mmol) in water (10 ml) was added dropwise a solution of NaOCl (available chrorine: minimum 5%, 4.9 ml) during a 20 minutes period while the pH was kept 7 to 8 with 2 N HCl. After stirring at room temperature for an hour, the reaction mixture was acidified to pH 2 by addition of 2 N HCl and then extracted with ether The ether extract was dried over $MgSO_4$ and evaporated under a reduced pressure to give a residue (775 mg), which was purified by column chromatography on silica gel, eluted with methanol-$CHCl_3$ (3:97) to afford 4-chloro-4,4-dimethoxycarbonyl-(3R)-methylbutyric acid (15, 504 mg, 68%) as an oil: $[\alpha]_D^{25}$ +0.67° (*c* 1.2, methanol), ^{1}H-NMR (200 MHz, $CDCl_3$) δ 9.2 (1H, br), 3.8 (6H, s), 3.1 (1H, m), 2.76 (1H, dd, *J* = 16 and 2 Hz)), 2.26 (1H, dd, *J* = 16 and 10 Hz), 1.05(3H, d, *J* = 7 Hz).

Dimethyl 2-Chloro-2-((2-hydroxycarbamoyl-(1R)-methyl)ethyl)malonate (16) and Methyl 3-Chloro-hydroxy-(4R)-methyl-2.6-dioxo-3-piperidinecarboxylate (17)

To a solution of 15 (504 mg, 2 mmol) in CH_2Cl_2 (10 ml) was added *N*-hydroxysuccinimide (230 mg, 2 mmol), and the mixture was stirred at room temperature for an hour. To

the resulting solution was added dicyclohexylcarbodiimide (412 mg, 2 mmol) at 0℃ and the mixture was successively stirred at 0℃ for 5 minutes and at room temperature for 6 hours The reaction mixture was filtered, and filtrate was concentrated to dryness under a reduced pressure to give *N*-hydroxysuccinimide ester of 15 (700 mg) as a crude product. To a solution of hydroxylamine hydrochloride (167 mg, 2.4 mmol) in water (2 ml) was added 1 N NaOH (3 ml, 3 mmol) at 0℃ with stirring. The resulting solution was added dropwise to a solution of 4-chloro-4,4-dimethoxycarbonyl-(3R)-methylbutyric acid succinimide ester (700 mg) in ethanol (5 ml) at 0℃. The mixture was successively stirred at room temperature overnight and at 80℃ for 2 hours. The reaction mixture was acidified to pH 2 with 1 N HCl and then extracted with ethyl acetate. The ethyl acetate extract was washed with water, dried over $MgSO_4$ and concentrated to dryness under a reduced pressure to give a residue (477 mg), which was purified by column chromatography on silica gel, eluted with methanol-$CHCl_3$ (1:19) to afford a mixture of 16 and 17 (250 mg), 17 (145 mg): FAB-MS, *m/z* 240, 241 (M^+-H_2O+Na), $(\alpha)_D^{22}$ -13.6° (*c* 1, methanol), ^{1}H-NMR (200 MHz, $CDCl_3$) δ 3.88 (3H, s), 3.10 (1H, m), 2.82 (2H, m), 1.05 (3H, d, J = 7 Hz).

2-Hydroxy-2-(hydroxycarbamoyl)-(3R)-methylglutaric Acid (WS1358A1, 1a)

To a mixture of 16 and 17 (27 mg) was added 1 N NaOH (1 ml), and the mixture was stirred at room temperature for 2 hours. The reaction mixture was concentrated to dryness under a reduced pressure to give a product which contained WS1358A1 (1a) and its epimer, A1' (1b) at a ratio of 7:2. The ^{1}H-NMR spectrum and the retention time on HPLC of the synthetic WS1358A1 was consistent with those of natural WS1358A1.

Biological evaluation

Inhibitory activity (IC_{50}) of the synthetic compounds against porcine DHP was measured by the method described in the preceding chapter.

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Chapter IV Biological Properties of WS1358A1

IV-1 *In vitro* properties of WS1358A1

WS1358A1 and B1 were found to inhibit renal DHP from several species of animal as shown in Table 1. Especially, WS1358A1 was more potent than cilastatin against all of the DHPs tested. In spite of their potent inhibitory activity against DHP, WS1358A1 and B1 did not inhibit carboxypeptidase A (CPase A) and leucineaminopeptidase (LAP) at a concentration of 100 μM. Therefore, WS1358 compounds were specific inhibitors against DHP.

It was necessary to purify porcine renal DHP in order to examine the inhibitory mechanism of WS1358A1 against this enzyme. In our procedure of purification, the steps of acetone precipitation, extensive washing with buffer and isoelectric precipitation previously reported have been eliminated to save labor and time.

A summary of the typical purification is shown in Table 2. From 94 g of porcine kidney cortex, 7.8 g of protein homogenate was obtained. The complete purification from this homogenate yielded 0.6 mg of DHP which was apparently homogenous and had a molecular weight of about 50,000 as judged by the SDS-polyacrylamide gel electrophoresis (data not shown). The over-all purification produced an enzyme approximately 1,800 times more active than the starting homogenate.

Table 1. IC_{50} values of WS1358A1, B1 and cilastatin against various peptidases.

Peptidase	IC_{50} (μM)		
	A1	B1	Cilastatin
Renal DHP:			
Porcine	0.003	0.60	0.13
Rabbit	0.04	2.7	0.075
Mouse	0.01	1.6	0.25
Rat	0.008	1.1	0.20
CPase A	$>10^2$	$>10^2$	$>10^2$
LAP	$>10^2$	$>10^2$	$>10^2$

Enzymes were assayed using synthetic peptides as substrate. Enzyme activity was determined by measuring the decline of absorbance in the presence or the absence of an inhibitor. Inhibition percent was calculated as described in the text.

Table 2. Purification of porcine renal dehydropeptidase.

Fraction	Total protein (mg)	Total activity[a] (U)[a]	Activity recovered (%)	Specific activity (U/mg)	Purification (fold)
Homogenate	7,800	78	100	0.010	1
Solubilized	2,688	97	124	0.036	3.6
50～70% $(NH_4)_2SO_4$	128	53.4	69	0.42	42
HPLC	12.2	22.6	29	1.8	180
Affinity chromatography	0.6	11.0	14	18.3	1,830

[a] All fractions were assayed using 50 μM GDP as substrate in 25 mM Tris-HCl buffer (pH 7.7).

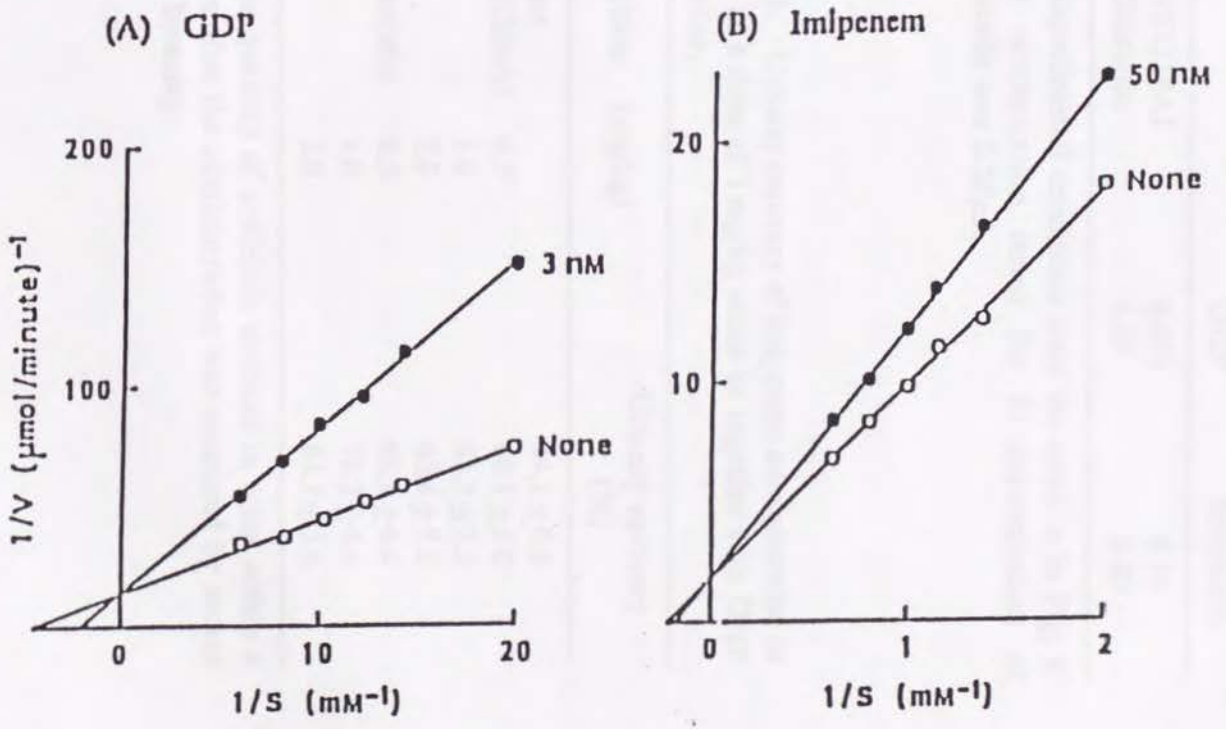
Before measuring the kinetic parameters for enzyme-catalyzed hydrolysis, a linearity between the purified enzyme concentration and enzyme activity against GDP was established over the enzyme concentration range of 0.05 through 10 μg/ml. The kinetic parameters were then determined for GDP and imipenem in the absence or the presence of inhibitor, using the standard graphical technique of Lineweaver-Burk. Typical reciprocal plots for GDP and imipenem are shown in Fig. 1. The following kinetic parameters of the purified enzyme could be calculated for the two substrates GDP (K_m = 0.3 mM, V_{max} = 800 μmol/min per mg) and imipenem (K_m = 7.7 mM, V_{max} = 23 μmol/min per mg).

WS1358A1 as well as cilastatin showed a competitive inhibition and the inhibition constants for WS1358A1 using GDP and imipenem as substrates are K_i, 2×10^{-9}, 1.6×10^{-7} M, respectively. The inhibition constants for WS1358A1 compared with those of cilastatin are listed in Table 3.

IV-2 In vivo properties of WS1358A1

As shown in Table 4, the mean urinary recovery percents of imipenem intraveneouly administrated to mice at a dose of 1 mg/kg without DHP inhibitor were 44.1%. However, when WS1358A1 was co-administrated at a dose of 1 mg/kg with imipenem (1:1), the urine recovery percents increased up to 63.8%. The urinary recovery of imipenem was also signifi-

Fig. 1. Lineweaver-Burk plot of porcine renal dehydropeptidase-catalyzed hydrolysis in the presence or absence of WS1358A1.



The DHP-catalyzed hydrolysis rates were measured at 37°C in 25 mM Tris-HCl buffer (pH 7.7). The enzyme was mixed with an inhibitor and pre-warmed at 37°C for 1 minute before adding the substrate. Velocity is expressed as μmol/minute. Each point shows the mean of three to four determinations.

Table 3. Inhibition constants of DHP inhibitors.

Inhibitor	*Ki* (μM)	
	GDP	Imipenem
WS1358A1	0.002	0.16
Cilastatin	0.09	0.30

Experimental conditions were the same as in Fig. 1. The concentration tested for *Ki* determination of cilastatin was 0.26 μM.

Table 4. Urinary recovery of imipenem administrated to mice at a dose of 1 mg/kg alone or together with DHP inhibitor.

Inhibitor	(mg/kg)	Urinary recovery (%)
None		44.1±0.6
WS1358A1	0.5	66.1±3.0
	1.0	63.8±7.3
	2.0	65.4±9.5
Cilastatin	0.5	68.9±6.4
	1.0	70.2±6.0
	2.0	63.1±5.6

The quantity of antibiotic excreted in urine within 4 hours after the administration was measured by means of a bioassay.

cantly augmented at the combination ratios of WS1358A1 versus the antibiotic within a range of 0.5:1 to 2:1 by weight. A similar effect was observed when cilastatin was used as inhibitor in place of WS1358A1. Therefore, the effect of WS1358A1 in mice on urinary recovery of imipenem resembles that of cilastatin when co-administrated with imipenem to human volunteers[1].

Although carbapenem antibiotics show strong antimicrobial activity *in vitro*[2], their ability to protect mice from experimental infection[3] is somewhat weak because of their susceptibility to inactivation by renal DHP. As shown in Table 5, imipenem showed no protective activity when it was administrated alone at the doses of 0.4 - 2.0 mg/kg. In this case, no mice survived at day 5 after challenge. But, it showed an improved protective activity when WS1358A1 was co-administrated with the antibiotic at the combination ratio of 1:1. Two and four of five mice were cured at the doses of 0.4 and 2.0 mg/kg, respectively. The similar result was obtained when WS1358A1 was replaced by cilastatin.

Accordingly the improved protective effect of DHP inhibitors in experimental infection related to the augmentation of urinary recovery by the inhibitors.

IV-3 Summary

The co-administration of WS1358A1 with a carbapenem

Table 5. Protective effect of imipenem with or without DHP inhibitor on experimental infection[a].

Drug[b]	Dose (mg/kg)	Survived/treated[c]
Saline		0/5
Imipenem	0.4	0/5
	2.0	0/5
	10.0	3/5
Imipenem + WS1358A1[d]	0.4	2/5
	2.0	5/5
	10.0	5/5
Imipenem + cilastatin[d]	0.4	2/5
	2.0	4/5
	10.0	5/5

[a] ICR mice were intraperitoneally infected with 4×10^8 *Staphylococcus aureus* 47.

[b] Drugs were subcutaneously administrated at 1 hour after the challenge.

[c] Mortalities were determined at day 5 after the infection.

[d] Co-administration at a combination ratio of 1:1 (w/w).

antibiotic imipenem increased the urinary recovery of the antibiotic and improved the protective effect on experimental infections. The efficacy of WS1358A1 is comparable to that of cilastatin[1,4]. The author can attribute the *in vivo* effects of WS1358A1 to the potent inhibitory activity of the compound on DHP by which carbapenem antibiotic is inactivated.

WS1358A1 should be considered in combination therapy like imipenem/cilastatin.

Experimental

Enzyme assay

Renal DHPs from several species of animal were partially purified by the method described below for porcine renal DHP except that procedures following the $(NH_4)_2SO_4$ precipitation were omitted.

The effect of WS1358 compounds against the partially purified DHPs was measured by a method similar to that used for porcine DHP. Moreover, to ascertain the specificity of inhibitory activity, the effects of WS1358 compounds against metallo-enzymes other than DHP, such as carboxypeptidase A (CPase A) and leucineaminopeptidase (LAP) were determined. CPase A was assayed by using carbobenzoxyglycyl-L-phenylalanine as substrate. The activity was determined by observing the decline in absorbance at 224 nm. LAP was assayed by using L-leucine-*p*-nitroanilide as substrate. The activity was determined by observing the decline in absorbance at 405 nm. Inhibitory effects on the two enzymes were calculated in the same manner as those on DHP described in the chapter II.

Purification of porcine renal DHP

Purification of DHP from porcine kidney cortex to apparent homogeneity as judged by SDS polyacrylamide gel electrophoresis was carried out using the procedures slightly different from those previouly reported[5-7].

The cortex was sliced from the intact kidney and homogenized in 25 mM sodium phosphate buffer (pH 8.0) at 4℃ in a Polytron homogenizer. The homogenate was adjusted to 20% butanol by the addition of butanol which had been chilled to -20℃. Solubilization of the enzyme was achieved by stirring the mixture at 4℃ overnight. After dialysis for 24 hours against several changes of distilled water, the enzyme activity was concentrated by precipitation between 50 - 70% $(NH_4)_2SO_4$. This partially purified enzyme was used to search for DHP inhibitors from fermentation broths. Futher purification of renal DHP was achieved by means of a HPLC and an

affinity column chromatography. HPLC of the $(NH_4)_2SO_4$ fraction was carried out on a TSK SW3000 column using a Waters model 6000 HPLC chromatograph with a Waters moel 440 absorbance detector and a Hitachi 200 recorder. Affinity column chromatography was performed using a procedure reported by Kropp *et al*[6]. The DHP inhibitor, cilastatin, was coupled to cyanogen bromide-activated Sepharose 4B following the manufacturer's instruction. The activity of the enzyme at various stages of purification was determined by the method described below.

Enzyme kinetic study

The activity of DHP was determined by measuring the rate of enzyme-catalyzed hydrolysis of the unsaturated dipeptide glycyldehydrophenylalanine (GDP) at 37℃. The fall in absorbance of a solution of 5×10^{-5} M of the peptide in 25 mM Tris-HCl buffer (pH 7.7) was measured at 275 nm. Protein concentrations were determined by the method of Bradford[8]. Enzyme units are expressed as micromoles of substrate hydrolyzed/minute, and specific activity is expressed as micromoles of substrate hydrolyzed/minute/mg of protein under the assay condition described above.

The rates of enzyme-catalyzed hydrolysis of GDP and imipenem in the absence or the presence of an inhibitor were measured in the similar manner. The enzyme concentrations employed in the reactions were 0.1 μg/2.0 ml for GDP and 44 μg/2.0 ml for imipenem.

Dertermination of urinary recovery

For each compound or combination, a group of four mice were given 1 ml water orally and administrated by intravenous injection. Urine was collected over 0 - 4 hours period. Antibiotic concentration in urine samples were determined by microbiological assay using *Bacillus subtilis* ATCC 6633 against standard of imipenem prepared in 0.05 M phosphate buffer (pH 7).

Experimental infection

Female Jcl:ICR mice, 4 weeks of age, were purchased from Shizuoka Laboratory Animal Center. Hamamatsu, Japan. Groups of five mice were intraperitoneally injected with 10^8 *Staphylococcus aureus* 47 suspended in 5% mucin solution. Combined therapies were subcutaneously administrated at 1 hour after the infection. Mortalities were determined after the observation for 5 days.

Materials

CPase A from bovine pancreas (Type II-DEF) and LAP from porcine kidney microsomes (Type IV-S) were purchased from Sigma Chemical Co. Carbobenzoxyglycyl-L-phenylalanine and L-leucine-*p*-nitroanilide were purchased from Peptide Institute, Inc., Osaka, Japan.

GDP[9] were synthesized by our chemists. Imipenem and cilastatin were purified from Zienam. CNBr-activated Sepharose 4B was purchased from Pharmacia.

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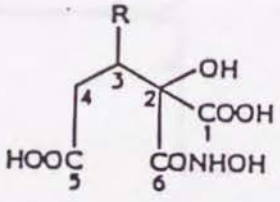
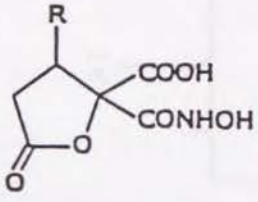
Chapter V Chemical Transformation and Total Synthesis of WS1358A1

V-1 Chemical transformation

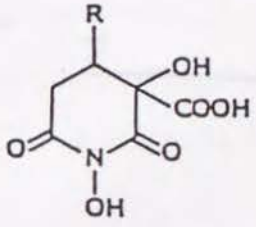
During the course of studies on these compounds, we found that A1 suffers chemical transformations to butyrolactone A2 (3) and *N*-hydroxyglutarimide A3 (5), which are readily reconverted to the original compound A1. As described in the chapter II, these transformations were also found to expedite the purification of this series of natural products.



1 R=CH_3
2 R=H



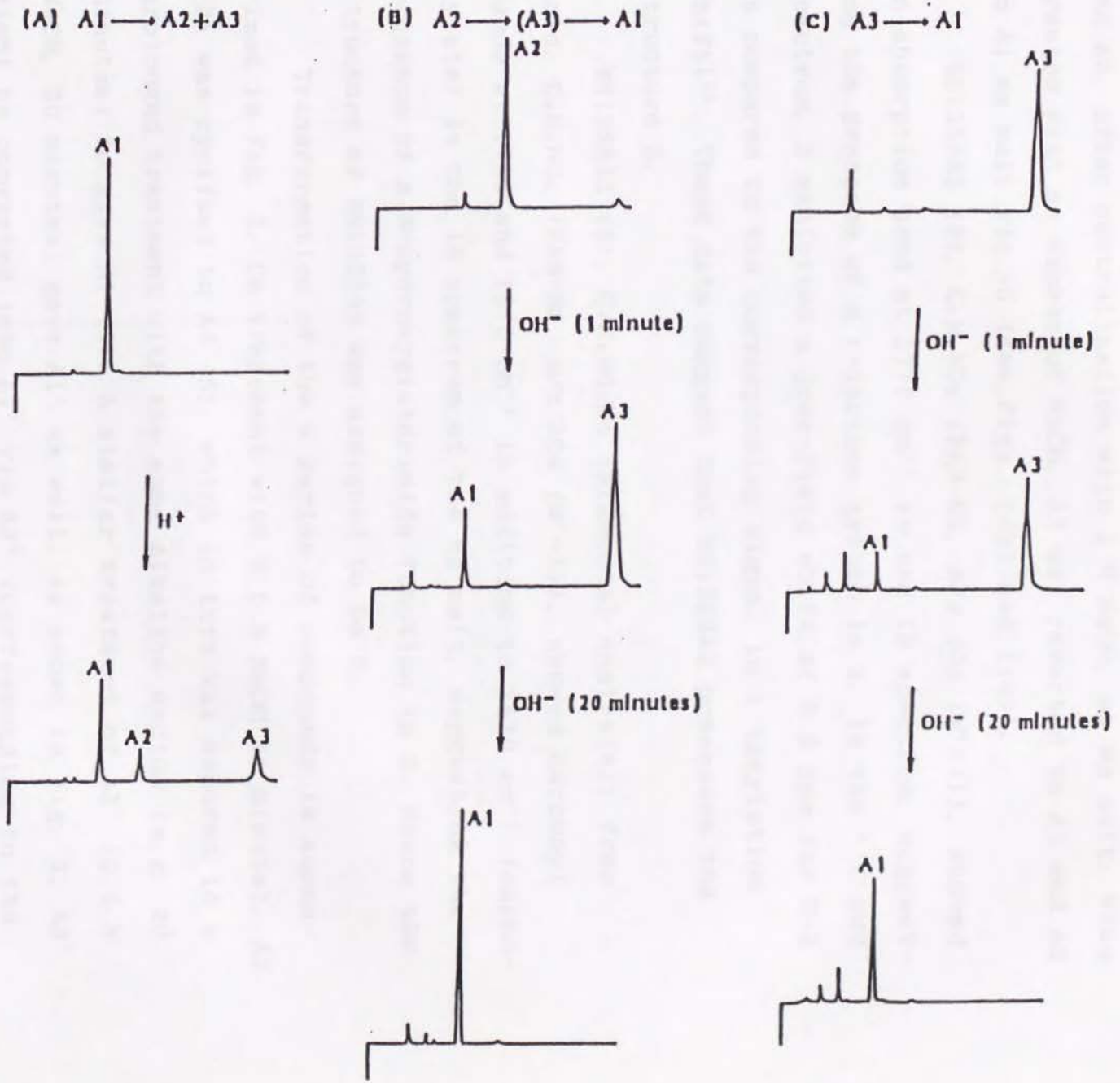
3 R=CH_3
4 R=H



5 R=CH_3
6 R=H

WS1358A1 (1, Na salt) was converted to the free acid by treatment with Dowex 50WX2 (H^+) and lyophilized to give a mixture of A1, A2 (3) and A3 (5) (see Fig. 1(a) for HPLC analysis). Separation by the preparative HPLC using YMC ODS-

Fig. 1. Chemical transformation of natural WS1358A series: HPLC analysis.



5 reverse-phase column (20 mM $NH_4H_2PO_4$ - 1 mM Bu_4NOH (pH 4)), followed by acidification (Dowex 50WX2 (H^+)) and purification (Diaion SP-207 chromatography) gave A2 as free acid and A3, after neutralization with 1 N NaOH, as Na salt. When treated with an excess of NaOH, A3 was reverted to A1 and A2 to A1 as well *via* A3 (see Figs. 1(b) and 1(c)).

WS1358A2 (3), $C_7H_9NO_6$ (FAB-MS, *m/z* 204 (M^++1)), showed an absorption band at 1770 cm^{-1} in the IR spectrum, suggesting the presence of a γ-lactone system in 3. In the ^{13}C NMR spectrum, 3 exhibited a down-field shift of 5.4 ppm for C-2 as compared to the corresponding signal in 1 (acylation shift)[1]. These data suggest that WS1358A2 possesses the structure 3.

WS1358A3 (5), $C_7H_8NO_6Na$ (elemental analysis); free acid, $C_7H_9NO_6$ (FAB-MS, *m/z* 204 (M^++1)), showed carbonyl bands at 1730 and 1670 cm^{-1} in addition to 1630 cm^{-1} (carboxylate) in the IR spectrum of the Na salt, suggesting the presence of a *N*-hydroxyglutarimide function in 5. Hence the structure of WS1358A3 was assigned to be 5.

Transformation of the A series of compounds is summarized in Fig. 2. On treatment with 0.5 N NaOH (1 minute), A2 (3) was cyclized to A3 (5), which in turn was exposed to a prolonged treatment with the same alkaline medium (*e.g.* 20 minutes) to give A1 (1). A similar treatment of A3' (0.5 N NaOH, 20 minutes) gave A1' as well. As shown in Fig. 2, A3' might be converted into A1' *via* A2' (corresponding to the diastereoisomer of A2) which could not be isolated in the

Fig. 2. Proposed pathway of chemical transformation of WS1358A series.

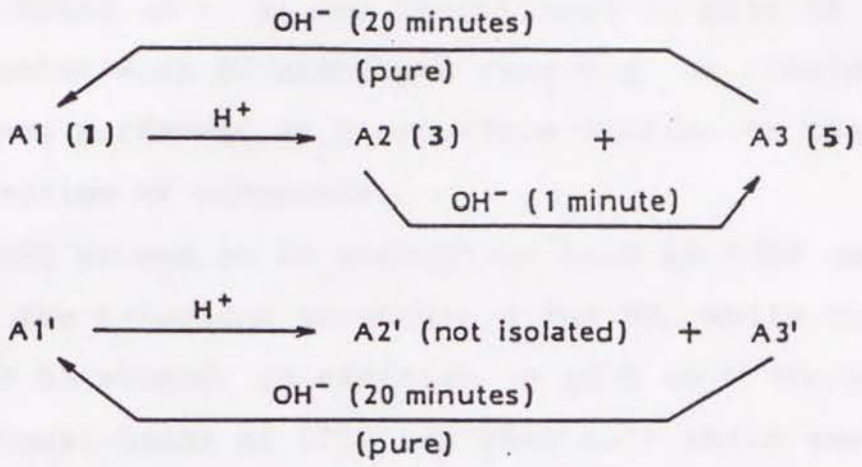
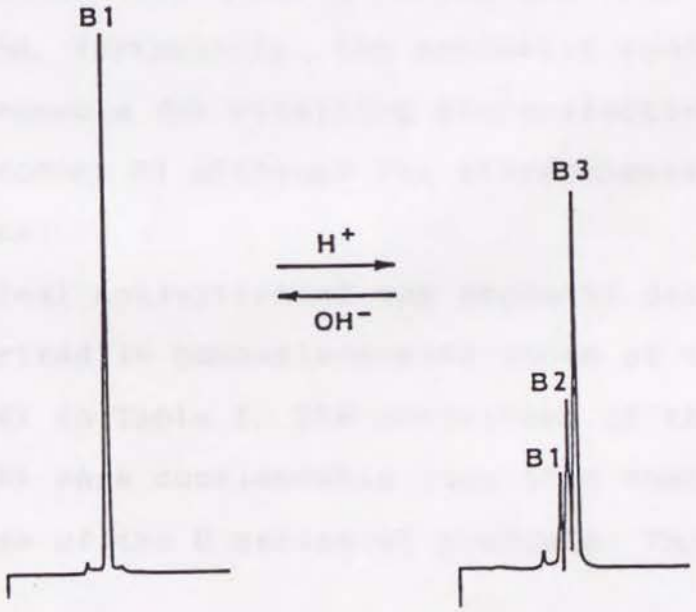


Fig. 3. Chemical transformation of natural WS1358B series: HPLC analysis.



synthesis described above.

The minor product B1 (2) from the natural source was found to show a similar behavior. Thus, after acidification with Dowex 50WX2 (H^+), B1 was lyophilized to give B2 (4) and B3 (6) together with B1 unchanged (see Fig. 4). Isolation of B2 and B3 was performed by a procedure similar to that used for the A series of compounds.

WS1358B2 showed an IR absorption band at 1780 cm^{-1}, indicating the γ-lactone structure 4 for B2, while the IR spectrum of B3 showed, in addition to 1680 cm^{-1} (carboxylic acid), carbonyl bands at 1730 and 1640 cm^{-1} which were assignable to the carbonyls of the *N*-hydroxyglutarimide structure, leading to the structure 6 for B3. The other physical data of B2 and B3 are recorded in Experimental. On treatment with 0.5 N NaOH, B2 and B3 showed a behavior similar to those of A2 and A3, respectively, and finally the two compounds reverted to B1 (2).

Each product of both A and B series was thus chemically characterized and, fortunately, the synthetic route described above was amenable for obtaining stereoslectively the major natural product A1 although its stereochemistry remained equivocal.

The biological activities of the products described above are summarized in comparison with those of the natural A1 (1) and B1 (2) in Table 1. The activities of the natural A2 (3) and A3 (5) were considerably less than that of A1 and this was the case of the B series of products. The synthetic

compounds A1, A2, A3 and B1 (all racemates) were approximately 2-fold less active than the corresponding natural products, respectively. The diastereoisomer A1' and A3' were considerably less active even when compared with the synthetic A1 and A3, respectively.

Table 1. Inhibitory activity (IC_{50}) of synthetic and natural WS1358 compounds against porcine DHP.

Compound	IC_{50}(ng/ml)	
	Synthetic	Natural
A1 (1)	1.5	0.83
A1'	230	nd
A2 (3)	16	10
A3 (5)	78	45
A3'	4,300	nd
B1 (2)	340	150
B2 (4)	nd	2,000
B3 (6)	nd	>10,000

nd: Not determined.

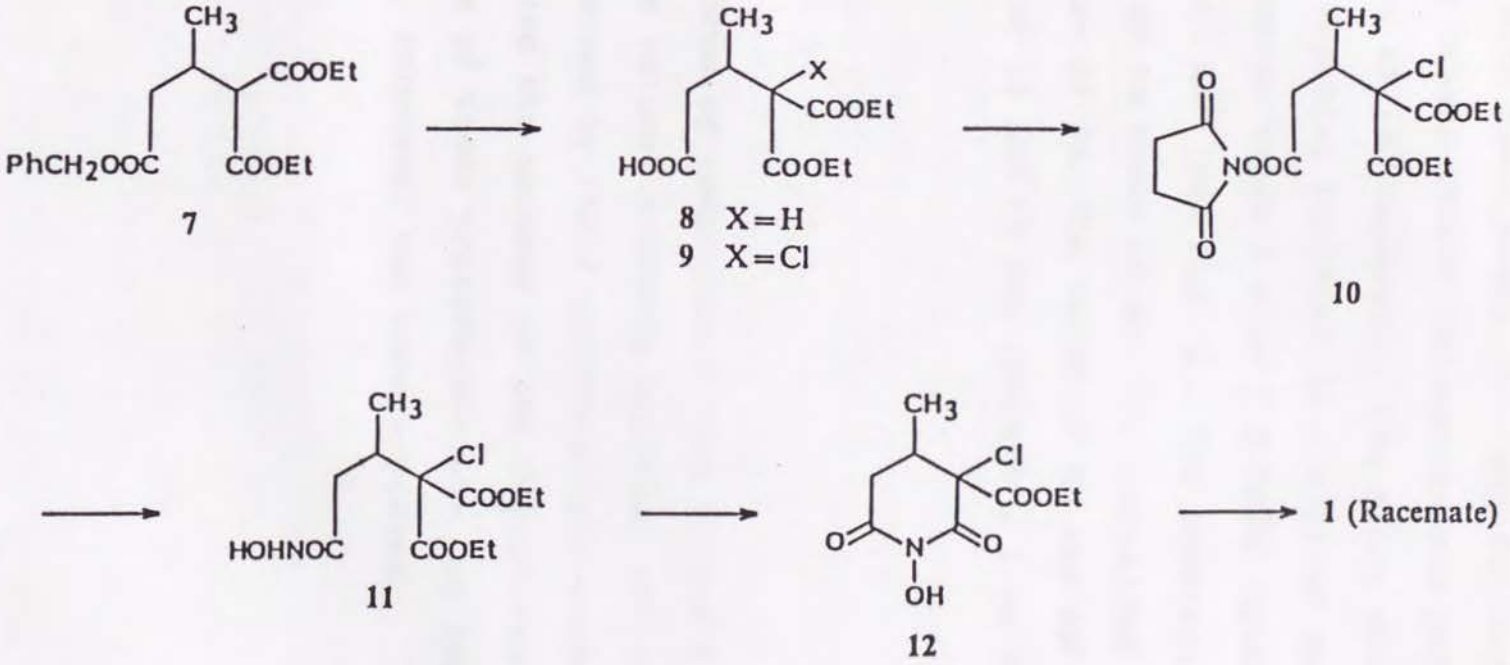
V-2 Total synthesis of WS1358A1

The author found in the conversion of A3 to A1 that the hydrolysis was regiospecific to yield A1 almost as a single product and, therefore, we anticipated that a stereoselective synthesis of A3 could be entirely corresponding to a regio- and stereoselective synthesis of A1. Thus, I undertook the synthesis of A1 according to the synthetic route

outlined in Fig. 4. I envisioned that, in this synthesis, the cyclization of 11 to 12 would proceed thermodynamically, resulting in a stereoselective synthesis of 12. Hydrolysis of 12 would then stereoselectively afford the final product 1 (racemate) as described above, although it was ambiguous at that time whether the desired configuration would be obtained.

The starting material 7, prepared by Michael condensation[2] of benzyl crotonate and diethyl malonate, was hydrogenolysed and the resulting carboxylic acid 8 was chlorinated with NaOCl to give chloride 9. Activation of the carboxylic acid in 9 with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide, followed by treatment with hydroxylamine produced a mixuture of hydroxamic acid 11 and *N*-hydroxyglutarimide 12 together with other minor products. It was clear that, in the above reactions, 12 was formed by cyclization of 11 (see below for a corroborative evidence). The separation of 11, 12, and the minor products at this stage was found to be difficult, because 11 was partially cyclized to 12 during the period of purification by chromatography on silica gel. Therefore, the mixture was directly subjected to the next reaction and treated with 1 N NaOH. The reaction mixture was acidified using Dowex 50WX2 (H^+) and lyophilized to give a crude mixture, which was applied to a Diaion SP-207 column chromatography to give pure A3 (5, racemate) as the first eluent. Later fractions containing A2 and the minor products were further purified by another SP-207 chromatography,

Fig. 4. Improved synthesis of WS1358A1 racemate.



followed by preparative HPLC (YMC ODS-5) to give a partially purified A2 (3) and one of the minor products. The product A2 was treated with Dowex 50WX2 (H^+) and followed by further purification by Diaion SP-207 chromatography and lyophilization to afford A2 (3, racemate). The minor product isolated as above was also purified in a similar manner and, after neutralization to pH 6 with 1 N NaOH, lyophilized to give pure product A3' (see Fig. 2). The spectral data of A3' was quite similar to those of A3 (5), revealing that A3' is a diastereoisomer of A3. The ratio of A3 and A3' just after the hydrolysis of 11 and 12 was about 5 : 1 on HPLC.

V-3 Summary

The structures of compounds 3 (and 4) and 5 (and 6) derived from the natural products WS1358A1 (1) (and B1 (2)) have been determined by their spectroscopic evidence. HPLC analyses clarified the pathway of the transformation. By taking advantage of these transformations, an improved synthesis of A1 (1, racemate) has been achieved.

Experimental

General

IR spectra were recorded on a Jasco A-120 spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AM400 spectrometer. Mass spectra were determined with a VG ZAB-SE spectometer. Analytical HPLC was performed on a Hitachi 655 liquid chromatography using a YMC ODS-5 column (4.6 X 250 mm) and preparative HPLC was carried out using a YMC ODS-5 column (20 X 250 mm).

WS1358A2 (3) and A3 (5)

WS1358A1 (1, Na salt, 100 mg) was dissolved in water (2 ml) and passed through a column of Dowex 50WX2 (H^+, 3 ml) and eluted with water (10 ml). The eluate was adjusted to pH 1.8 with 6 N HCl and lyophilized to give a powder (76 mg, see Fig. 1 (a) for HPLC analysis). Preparative HPLC of this mixture was carried out on YMC ODS-5 using a buffer of 20 mM $NH_4H_2PO_4$ - 1 mM Bu_4NOH (pH 4) as eluant. The fraction containig A2 was purified by chromatography on Diaion SP-207 (7.5 ml) eluting with water and the eluate was passed through a column of Dowex 50WX2 (H^+, 7 ml) and eluted with water. The eluate was lyophilized to give A2 (3, 24 mg) as the free acid: FAB-MS, *m/z* 204 (M^++1); IR (KBr) cm^{-1} 1780-1640 (broad); ^{1}H NMR (D_2O) δ 3.16 (1H, m), 2.87 (1H, dd, J = 18 and 8.5 Hz), 2.49 (1H, dd, J = 18 and 8 Hz), 1.23 (3H, d, J = 7 Hz); ^{13}C NMR (D_2O) δ 180.6 (s), 171.8 (s), 168.6 (s), 91.2 (s), 39.6 (d), 37.7 (t), 17.9 (q).

The Na salt of A2 was prepared by neutralization of a solution of the free acid in water to pH 7 with 1 N NaOH and subsequent lyophilization: IR (KBr) cm^{-1} 1770, 1650, 1600; ^{1}H NMR (D_2O) δ 3.08 (1H, m), 2.82 (1H, dd, J = 18 and 8 Hz), 2.40(1H, dd, J = 18 and 7 Hz), 1.15 (3H, d, J = 7 Hz); ^{13}C NMR (D_2O) δ 181.9 (s), 174.4 (s), 170.4 (s), 93.4 (s), 38.9 (d), 38.5 (t), 18.4 (q).

The fraction containing A3 on the preparative HPLC was similarly purified. The eluate obtained by a Dowex 50WX2

column chromatography was neutralized to pH 7 with 1 N NaOH and lyophilized to give A3 (5, 32 mg) as the Na salt: IR (KBr) cm^{-1} 1730, 1670, 1630; ^1H NMR (D_2O) δ 2.86 (1H, dd, J = 17.5 and 5 Hz), 2.76 (1H, dd, J = 17.5 and 9.5 Hz), 2.62 (1H, m), 0.97 (3H, d, J = 6.5 Hz); ^{13}C NMR (D_2O) δ 177.4 (s), 174.2(s), 173.9 (s), 83.0 (s), 38.6 (t), 34.5 (d), 15.9 (q).

The free acid was prepared by treatment of a solution of the Na salt in water with Dowex 50WX2 (H^+) and subsequent lyophilization: FAB-MS, m/z 204 (M^++1); IR (KBr) cm^{-1} 1720-1620 (broad); ^1H NMR (D_2O) δ 2.83 (1H, dd, J = 17 and 5 Hz), 2.83-2.65 (2H, m), 0.96 (3H, d, J = 7 Hz); ^{13}C NMR (D_2O) δ 175.5 (s), 173.4 (s), 171.9 (s), 82.3 (s), 38.0 (t), 34.4 (d), 15.6 (q).

WS1358B2 (4) and B3 (6)

Compounds B2 and B3 were obtained in a manner similar to that for A2 and A3 as the free acids. For HPLC analysis, see Fig. 4. B2 (4): FAB-MS, m/z 190 (M^++1); IR (KBr) cm^{-1} 1780, 1730, 1680; ^1H NMR (D_2O) δ 2.80-2.71 (4H, m); ^{13}C NMR (D_2O) δ 181.1 (s), 173.2 (s), 168.9 (s), 88.6 (s), 31.9 (t), 30.1 (t). B3 (6): FAB-MS, m/z 190 (M^++1); ^1H NMR (D_2O) δ 3.04-2.88 (2H, m), 2.45 (1H, m), 2.26 (1H, m); ^{13}C NMR (D_2O) δ 175.1 (s), 173.8 (s), 172.3 (s), 79.3 (s), 31.0 (t), 30.0 (t).

Conversion of A2 (3) to A1 (1) *via* A3 (5)

A sample (5 mg) of 3 (free acid) was dissolved in 0.5 N NaOH (5 ml) and, after 1 minute, the solution was analyzed using HPLC. After being left at room temperature for 20 minutes, the solution was again subjected to HPLC analysis. The data were shown in Fig. 1 (b).

Conversion of A3 (5) to A1 (1)

A sample (5 mg) of 5 (free acid) was dissolved in 0.5 N NaOH (5 ml) and, after being left at room temperature for 1 and 20 minutes subjected to HPLC analysis. The data were shown in Fig. 1 (c).

Benzyl 4,4-Diethoxycarbonyl-3-methylbutyrate (7)

A solution of diethyl malonate (8.16 g, 0.051 mol) in benzene (30 ml) was added to a suspension of sodium hydride (60% dispersion in mineral oil, 0.2 g, 5 mmol) in benzene (30 ml) under nitrogen atmosphere. After the evolution of hydrogen ceased, a solution of benzyl crotonate (9 g, 0.051 mol) in benzene (30 ml) was added to the above mixture. The mixture was refluxed for 15 hours and, after cooling to room temperature, the reaction mixture was poured into ice water (200ml). The organic layer was separated and the aqueous layer was extracted with ether. The benzene and ether layers were combined, washed with water, and dried over $MgSO_4$. Filtration and evaporation gave a crude product (17 g) which was further evaporated under reduced pressure (2 mmHg) to give 7 as the residue: EI-MS, *m/z* 336 (M^+); ^1H NMR ($CDCl_3$) δ 7.35 (5H, m), 5.12 (2H, s), 4.19 (4H, q, J = 7 Hz), 3.41 (1H, d, J = 7 Hz), 2.75 (1H, m), 2.61 (1H, dd, J =16 and 5 Hz), 2.37 (1H, dd, J = 16 and 8.5Hz), 1.26 (6H, t, J = 7 Hz), 1.08 (3H, d, J = 7 Hz).

4,4-Diethoxycarbonyl-3-methylbutyric Acid (8)

A solution of 7 (3.36 g, 0.01 mol) in dioxane (50 ml) was hydrogenated over 10% Pd-C (0.5 g) under 5 atm pressure of hydrogen. After removal of the catalyst by filtration, the filtrate was concentrated to give an oily residue (2.43 g), which was purified by chromatography on silica gel (60 g) eluting with 5% methanol in chloroform to give 8 as an oil (2.11 g): EI-MS, *m/z* 247 (M^++1); ^1H NMR ($CDCl_3$) δ 4.21 (4H, q, J = 7 Hz), 3.42 (1H, d, J = 7 Hz), 2.76 (1H, m), 2.64 (1H, dd, J = 16 and 5 Hz), 2.38 (1H, dd, J = 16 and 8.5 Hz), 1.28 (6H, t, J = 7 Hz), 1.11 (3H, d, J = 7 Hz).

4-Chloro-4,4-diethoxycarbonyl-3-methylbutyric Acid (9)

NaOCl (available chlorine 5% minimum, 25 ml, 0.017 mol) was added dropwise (30 min) to a solution of 8 (3.69 g, 0.015 mol) in water (50 ml) with stirring under room tempe-

Benzyl 4,4-Diethoxycarbonyl-3-methylbutyrate (7)

A solution of diethyl malonate (8.16 g, 0.051 mol) in benzene (30 ml) was added to a suspension of sodium hydride (60% dispersion in mineral oil, 0.2 g, 5 mmol) in benzene (30 ml) under nitrogen atmosphere. After the evolution of hydrogen ceased, a solution of benzyl crotonate (9 g, 0.051 mol) in benzene (30 ml) was added to the above mixture. The mixture was refluxed for 15 hours and, after cooling to room temperature, the reaction mixture was poured into ice water (200ml). The organic layer was separated and the aqueous layer was extracted with ether. The benzene and ether layers were combined, washed with water, and dried over $MgSO_4$. Filtration and evaporation gave a crude product (17 g) which was further evaporated under reduced pressure (2 mmHg) to give 7 as the residue: EI-MS, *m/z* 336 (M^+); ^1H NMR ($CDCl_3$) δ 7.35 (5H, m), 5.12 (2H, s), 4.19 (4H, q, J = 7 Hz), 3.41 (1H, d, J = 7 Hz), 2.75 (1H, m), 2.61 (1H, dd, J =16 and 5 Hz), 2.37 (1H, dd, J = 16 and 8.5Hz), 1.26 (6H, t, J = 7 Hz), 1.08 (3H, d, J = 7 Hz).

4,4-Diethoxycarbonyl-3-methylbutyric Acid (8)

A solution of 7 (3.36 g, 0.01 mol) in dioxane (50 ml) was hydrogenated over 10% Pd-C (0.5 g) under 5 atm pressure of hydrogen. After removal of the catalyst by filtration, the filtrate was concentrated to give an oily residue (2.43 g), which was purified by chromatography on silica gel (60 g) eluting with 5% methanol in chloroform to give 8 as an oil (2.11 g): EI-MS, *m/z* 247 (M^++1); ^1H NMR ($CDCl_3$) δ 4.21 (4H, q, J = 7 Hz), 3.42 (1H, d, J = 7 Hz), 2.76 (1H, m), 2.64 (1H, dd, J = 16 and 5 Hz), 2.38 (1H, dd, J = 16 and 8.5 Hz), 1.28 (6H, t, J = 7 Hz), 1.11 (3H, d, J = 7 Hz).

4-Chloro-4,4-diethoxycarbonyl-3-methylbutyric Acid (9)

NaOCl (available chlorine 5% minimum, 25 ml, 0.017 mol) was added dropwise (30 min) to a solution of 8 (3.69 g, 0.015 mol) in water (50 ml) with stirring under room tempe-

rature. The pH of the mixture was reached to 7.0. After stirring for an additional 30 minutes, the reaction mixture was acidified to pH 2.0 with 1 N HCl and extracted with ether. The extract was washed with water, dried over $MgSO_4$ and evaporated to give a crude oil, which was purified by chromatography on silica gel (150 g) eluting with 5% methanol in chloroform to give 9 as an oil (3.56 g): FAB-MS, *m/z* 281, 283 (M^++1); IR ($CHCl_3$) cm^{-1} 1740, 1710; ^1H NMR ($CDCl_3$) δ 4.30 (2H, q, J = 7 Hz), 4.29 (2H, q, J = 7 Hz), 3.14 (1H, m), 2.87 (1H, dd, J = 16.5 and 2.5 Hz), 2.33 (1H, dd, J = 16.5 and 10Hz), 1.31 (3H, t, J = 7 Hz), 1.29 (3H, t, J = 7 Hz), 1.12 (3H, d, J = 7 Hz).

4-Chloro-4,4-diethoxycarbonyl-3-methylbutyric Acid Succinimide Ester (10)

To a mixture of 9 (2.81 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mmol) in methylene chloride (50 ml) was added dicyclohexylcarbodiimide (2.06 g, 10 mmol) and the mixture was stirred at room temperature for 15 hours. After removal of the precipitate by filtration, the filtrate was concentrated to give 10 as a crude oil (2.96 g), which was used for the next reaction without purification: ^1H NMR ($CDCl_3$) δ 4.30 (2H, q, J = 7 Hz), 4.29 (2H, q, J = 7 Hz), 3.13 (1H, m), 3.10 (1H, dd, J = 16.5 and 2.5 Hz), 2.85 (4H, s), 2.59 (1H, dd, J = 16.5 and 10 Hz), 1.30 (3H, t, J = 7 Hz), 1.29 (3H, t, J = 7 Hz), 1.15 (3H, d, J = 7 Hz).

Diethyl 2-Chloro-2-((2-hydroxyaminocarbonyl-1-methyl)ethyl)malonate (11) and Ethyl 3-Chloro-1-hydroxy-4-methyl-2,6-dioxo-3-piperidinecarboxylate (12)

A solution of hydroxylamine hydrochloride (834 mg, 12 mmol) in water (10 ml) was, after neutralizaion by adding 1 N NaOH (15 ml), added to a solution of 10 (2.96 g, 10 mmol) in ethanol (30 ml) with stirring at 0℃. The mixture was stirred at room temperature for 5 hours and at 80℃ for 2 hours. The reaction mixture was acidified to pH 2 with 1 N HCl, diluted with water (150 ml), and extracted with ethyl

acetate. The extract was washed with water, dried over $MgSO_4$, and evaporated to give a crude mixture (2.7 g), which was purified by chromatography on silica gel (90 g) eluting with 5% methanol in chloroform to give a mixture of 11 and 12 (1.78 g). A 100 mg sample of this mixture was subjected to a preparative TLC (silica gel plate, 0.5 mm) developing with 10% 2-propanol in benzene to give 11 (44 mg, oil) and 12 (45 mg, oil). 11 (major diastereoisomer): FAB-MS, *m/z* 296, 298 (M^++1) and 318, 320 (M^++Na); IR ($CHCl_3$) cm^{-1} 1740, 1665; ^1H NMR (CD_3OD) δ 4.28 (4H, q, J = 7Hz), 3.10 (1H, m), 2.47 (1H, dd, J = 14 and 2.5 Hz), 2.03 (1H, dd, J = 14, 11 Hz), 1.29 (6H, t, J = 7 Hz), 1.04 (3H, d, J = 7 Hz). 12 (major diastereoisomer): FAB-MS, *m/z* 250, 252 (M^++1) and 272, 274 (M^++Na); IR ($CHCl_3$) cm^{-1} 1750, 1695; ^1H NMR (CD_3OD) δ 4.35 (2H, q, J = 7 Hz), 3.11 (1H, m), 2.82 (2H, m), 1.31 (3H, t, J = 7 Hz), 1.04 (3H, d, J = 7 Hz); ^{13}C NMR (CD_3OD) δ 168.2, 166.3, 165.3, 75.2, 64.9, 37.1, 34.0, 14.7, 14.2.

Alkaline treatment of 11 and 12

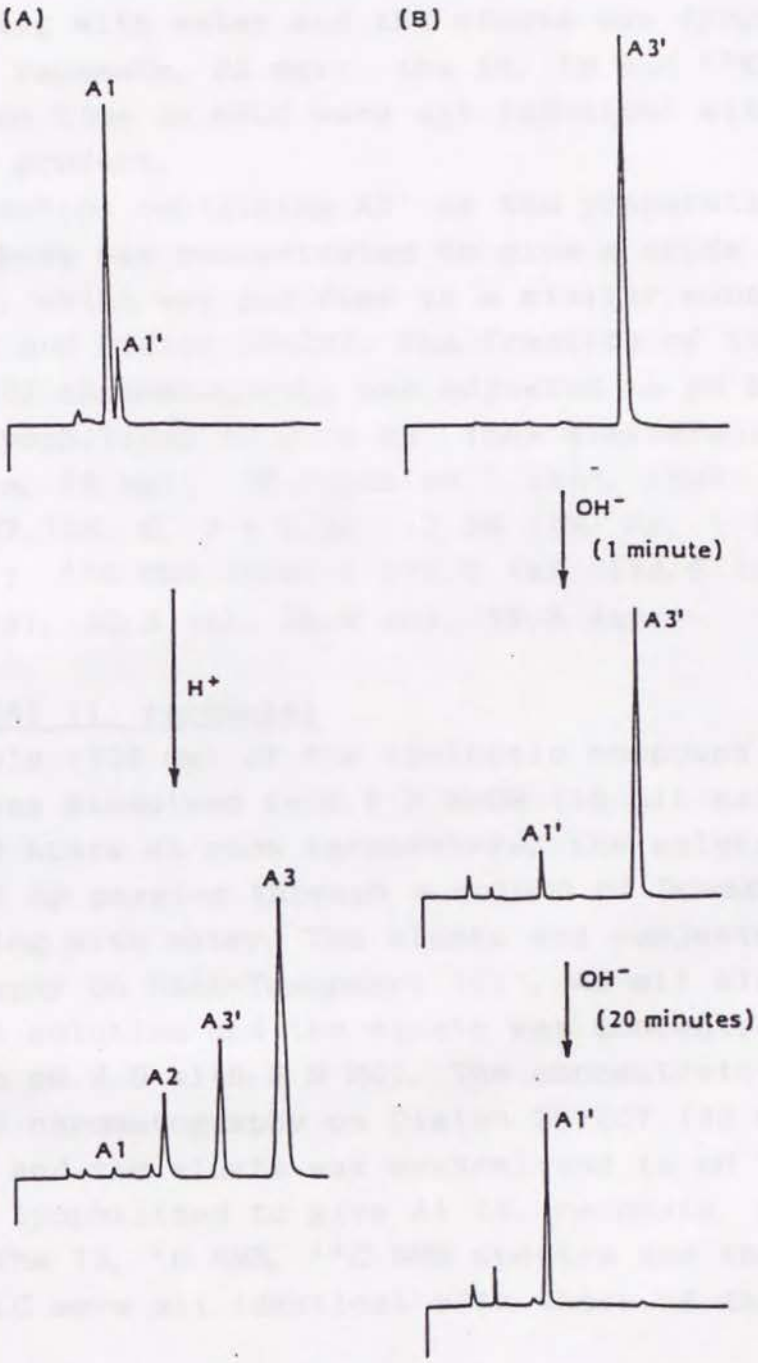
A mixture of 11 and 12 (400 mg) was dissolved in 1 N NaOH (8 ml) and stirred at room temperature for 3 hours. After neutralization to pH 7 with 1 N HCl, the mixture was analyzed by HPLC using a column of YMC ODS-5. The data were shown in Fig. 5 (a). The ratio of A1 (1) and its diastereoisomer was about 5 : 1.

The mixture obtained above was acidified by passing through a column of Dowex 50WX2 (H^+, 6 ml) eluting with water and the eluate was lyophilized to give a crude product containing A1, A2, A3, and their diastereoisomer (324 mg). This mixture was applied to a column of Diaion SP-207 (60 ml) eluting with water. The fractions containing A3 only were lyophilized to give pure A3 (5, racemate, 97 mg): The IR, ^1H and ^{13}C NMR spectra and the retention time on HPLC were all identical with those of the natural product.

The fractions containing A2 and A3' mainly were lyophilized to give a mixture of A2 and A3' (105 mg), which was subjected to preparative HPLC on YMC ODS-5 eluting with a

Fig. 5. Chemical transformation of synthetic WS-1358A series: HPLC analysis.

(A) A1 (A1') ⟶ A2 + A3 (A3'), (B) A3' ⟶ A1'.



buffer of 20 mM $NH_4H_2PO_4$ - 1 mM Bu_4NOH (pH 4) at a flow rate of 5.0 ml/minute. The fraction containing A2 was concentrated to give a crude product of A2 (42 mg), which was acidified by a Dowex 50WX8 (H^+) column (10 ml) eluting with water and the eluate was concentrated to about 2 ml. This concentrate was purified by chromatography on Diaion SP-207 (7 ml) eluting with water and the eluate was lyophilized to give A2 (3, racemate, 32 mg): the IR, ^1H and ^{13}C NMR and the retention time on HPLC were all identical with those of the natural product.

The fraction containing A3' on the preparative HPLC described above was concentrated to give a crude product of A3' (20 mg), which was purified in a similar manner using Dowex 50WX8 and Diaion SP-207. The fraction of the final Diaion SP-207 chromatography was adjusted to pH 6.2 (1 N NaOH) and lyophilized to give A3' (the diastereoisomer of A3, racemate, 16 mg): IR (KBr) cm^{-1} 1680, 1640; ^1H NMR (D_2O) δ 2.87 (2H, d, J = 9 Hz), 2.39 (1H, m), 1.00 (3H, d, J = 6.5 Hz); ^{13}C NMR (D_2O) δ 175.5 (s), 174.5 (s), 174.4 (s), 83.5 (s), 39.3 (t), 35.4 (d), 16.8 (q).

WS1358A1 (1, racemate)

A sample (500 mg) of the synthetic compound A3 (5, racemate) was dissolved in 0.5 N NaOH (10 ml) and allowed to stand for 3 hours at room temperature, the solution was then neutralized by passing through a column of Dowex 50WX2 (H^+, 6 ml) eluting with water. The eluate was subjected to chromatography on DEAE-Toyopearl (Cl^-, 45 ml) eluting with 0.02 M NaCl solution and the eluate was concentrated and adjusted to pH 2.0 with 6 N HCl. The concentrate was further purified by chromatography on Diaion SP-207 (60 ml) eluting with water and the eluate was neutralized to pH 7 with 1 N NaOH, then lyophilized to give A1 (1, racemate, Na salt, 213 mg): The IR, ^1H NMR, ^{13}C NMR spectra and the retention time on HPLC were all identical with those of the natural product.

Diastereoisomer A1' (racemate) of WS1358A1

A sample (20 mg) of A3' (racemate) described above was treated with 0.5 N NaOH in a manner similar to that for the conversion of A3 to A1, giving pure A1' (racemate, Na salt, 9 mg): IR (KBr) cm^{-1} 1650, 1620; ^1H NMR (D_2O) δ 2.76 (1H, m), 2.08 (1H, dd, J = 14 and 3 Hz), 1.99 (1H, dd, J = 14 and 11Hz), 0.87 (3H, d, J = 6.5 Hz); ^{13}C NMR (D_2O) δ 184.5 (s), 177.6 (s), 173.2 (s), 86.0 (s), 41.9 (t), 38.9 (d), 16.0 (q). For HPLC analysis, see Fig. 5 (b).

HPLC analysis

A Model 635 Hitachi liquid chromatograph was used in this study. A 15-cm TSK gel ODS 80TM column was used with a mobile phase consisting of 0.2 M $NH_4H_2PO_4$ (pH 4) containing 1 mM Bu_4NOH for WS1358A compounds. A 25-cm YMC ODS-5 column was used with a mobile phase of 0.1% TFA for WS1358B compounds. The flow rate was 1 ml/minute and solutes were detected by UV (210 nm). The retention times of A1, A2, A3, A1' and A3' were 7.15, 10.35, 19.55, 8.13 and 14.75 minutes, respectively. Those of B1, B2 and B3 were 3.73, 3.98 and 4.29 minutes, respectively.

Biological evaluation

Inhibitory activity (IC_{50}) of the compounds described above against porcine DHP was measured by the method described in the preceding chapter.

References

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Chapter VI Conclusion

Renal dipeptidase, dehydropeptidase-I (DHP, EC. 3. 4. 13. 11.) hydrolyzes the β -lactam ring of the carbapenems which are a new class of structurally novel β -lactam antibiotics. The low recovery of the carbapenems in the urine of laboratory animal and the low efficacy against experimental infection are attributed to the metabolism of the antibiotics by DHP.

Cilastatin, an inhibitor of DHP, was synthesized by Merck's researchers and has been clinically used in a new combination antimicrobial imipenem/cilastatin. In this combination, cilastatin has been proved to be effective in protecting imipenem, a derivative of thienamycin, from hydrolysis by DHP and restoring imipenem urinary recovery in human as well as in experimental animals.

No specific inhibitor against DHP other than cilastatin has been yet reported. During our screening program searching for novel enzyme inhibitors, the author found specific inhibitors against DHP, designated WS1358A1 and B1, from the fermented broth of *Streptomyces parvulus* subsp. *tochigiensis* No. 1358. The structure of WS1358A1 and B1 have been established to be 2-hydroxy-2-hydroxyaminocarbonyl-3-methylglutaric acid and 2-hydroxy-2-hydroxyaminocarbonylglutaric acid, respectively, on the basis of spectroscopic evidence and synthesis of the racemates.

To ascertain the possibility that WS1358A1 can be used as a counterpart in a combination therapy, a preliminary pharmacological evaluation in experimental animals was carried out.

The co-administration of WS1358A1 with a carbapenem antibiotic imipenem increased the urinary recovery of the antibiotic and improved the protective effect on experimental infection. The efficacy of WS1358A1 are comparable to that of cilastatin. The author can attribute the *in vivo* effects of WS1358A1 to the potent inhibitory activity of the compound on DHP by which carbapenem antibiotic is inactivated. WS1358A1 should be considered in a combination therapy with carbapenem antibiotics like imipenem/cilastatin.

During the course of studies on these compounds, the author found that A1 suffers chemical transformations to butyrolactone A2 and *N*-hydroxyglutarimide A3, which are readily converted to the original compound A1. These transformations were also found to expedite the purification of this series of natural products and a practical synthesis of WS1358A1 (racemate).

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